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**The role of Toll-like receptors in the development of  
immunological tolerance in neonates**

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*I dedicate this work, particularly to susceptible children at-risk who need a helping-hand and to all of us, who understand and support science at a very profound and fundamental level – the level of hope!*

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### 1.1 The *Hygiene hypothesis* in allergy and asthma development

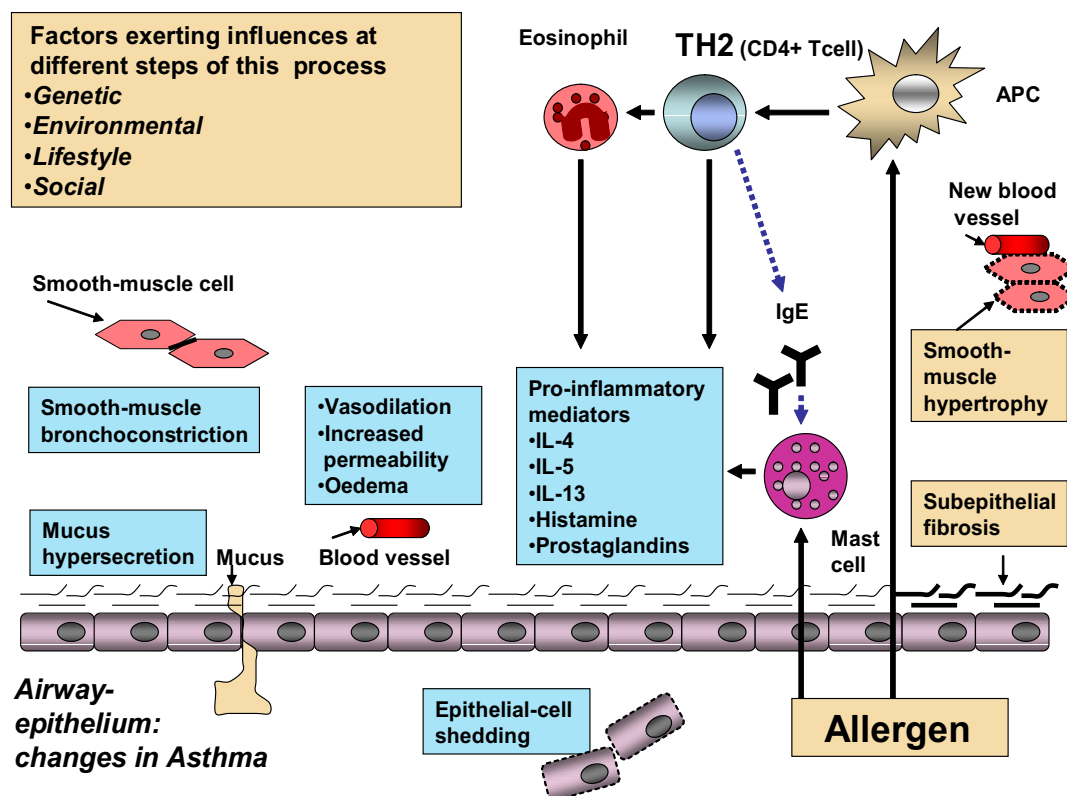
Based on epidemiological data, in latest '80s Strachan proposed the *Hygiene Hypothesis*. He suggested that infections and unhygienic contact with older siblings or through other exposures show protection from the development of allergic illnesses. This has evolved in various ways exploring the role of viral and bacterial infections, the impact of environmental exposure to bio-allergens such as microbes and their compounds, their effects on underlying responses of our innate and adaptive immunity. The relationship between a host's immune response, characteristics of the invading microorganism, the interactions between a genetic background and a range of exposures become more evident. All these could bring together to the clinical presentation of a complex disease so-called asthma and allergic illnesses (von Mutius, 2007). Allergic diseases are inflammatory disorders that develop on the basis of complex gene-environment interactions. The prevalence of allergies is progressively increasing and seems to be associated with modern lifestyle. Moreover, it was hypothesized that high living standards and hygienic conditions are correlated with an increased risk for the development of an allergic disease. The *Hygiene hypothesis* states that due to reduced exposure to microbial components, the proposed allergy-preventing potential of these factors is no more present in sufficient qualities and/or quantities, which leads to an imbalance of the immune system with a tendency to the development of allergic diseases. Meanwhile, numerous epidemiological studies are sustaining this theory, generating cellular and molecular designs for the underlying mechanisms that were then followed up by use of well-defined animal models for studying human allergies, which include changes in the balancing of T helper cell 1 ( $T_H1$ ), T helper cell 2 ( $T_H2$ ) and regulatory T cell ( $T_{reg}$ ) responses triggered by altered or missing innate immune cell activation. Consequently, a proper activation of cells of the innate immune system via their pattern recognition receptors (PRRs) has been demonstrated to play a crucial role in early determining of immune system and suppression of the development of  $T_H2$ -driven allergic immune responses. These processes start already *in utero*

and prenatal as well as early postnatal developmental stages seem to represent a *window of opportunity* for allergy-preventing environmental influences (Garn, 2007). Nevertheless, asthma, atopic dermatitis and atopic rhinoconjunctivities are three distinct conditions, which are characterized by inflammatory processes, with CD4<sup>+</sup>T helper (T<sub>H</sub>)-cell responses of the T<sub>H</sub>2-cell phenotype. Cytokines that are secreted by T<sub>H</sub>2 cells such as interleukins: IL-4, IL-5, IL-9 and IL-13, are important mediators of asthmatic and allergic inflammation, characterized by increased specific immunoglobulin E (IgE), mast-cell degranulation and eosinophil-mediated inflammation - against common, ubiquitous bio-allergens such as house dust mites, pollen or animal dander (Tab.1.1a).

Syndrome	Common allergens	Route of entry	Response
Systemic anaphylaxis	Drugs Serum Venoms Peanuts	Intravenous (either directly or following oral absorption into the blood)	Edema Increased vascular permeability Tracheal occlusion Circulatory collapse Death
Acute urticaria (wheal-and-flare)	Animal hair Insect bites Allergy testing	Through skin	Local increase in blood flow and vascular permeability
Allergic rhinitis (hay fever)	Pollens (ragweed, timothy, birch) Dust-mite feces	Inhalation	Edema of nasal mucosa Irritation of nasal mucosa
Asthma	Danders (cat) Pollens Dust-mite feces	Inhalation	Bronchial constriction Increased mucus production Airway inflammation
Food allergy	Tree nuts Peanuts Shellfish Milk Eggs	Oral	Vomiting Diarrhea Pruritis (itching) Urticaria (hives) Anaphylaxis (rarely)

**Tab.1.1a IgE-mediated reactions to extrinsic antigens.** The symptoms experienced by the patient can be very different depending on whether the allergen is injected, inhaled or eaten, and depending also on the dose of the allergen (according to: Janeway jr. 2006. Immunobiology - the immune system in health and disease, Handbook 6<sup>th</sup> Edition).

Once sensitized, re-exposure to allergen induces an inflammatory reaction of the affected organ - the bronchial system, the nasal and ocular mucosa, or the skin. In the airways of individuals with acute asthma diagnosis, this inflammatory response results in airway-narrowing because of mucosal edema, smooth-muscle constriction, mucus hypersecretion and epithelial-cell detaching process, whilst chronic inflammation - chronic asthma diagnosis - might lead to permanent structural changes, such as smooth-muscle hypertrophy and subepithelial fibrosis (Fig.1.1), (Devereux, 2006).



**Fig.1.1 Immunological pathways present in asthmatic airway-epithelium.** Asthma is characterized by inflammatory processes with CD4<sup>+</sup>T responses of the T<sub>H</sub>2-cell phenotype. T<sub>H</sub>2 – type cytokines, important mediators of asthmatic and allergic inflammation such as IL-4, IL-5, IL-9, IL-13 induce increasing of immunoglobulin E (IgE) level, eosinophilic cell number, respective mast-cell degranulation (according to: Devereux, 2006. The increase in prevalence of asthma and allergy: food for thought. Nature Reviews Immunology, 6: 869-874)

At present, atopic disease are the most common chronic illnesses in children living in the industrialized world. In general, asthma and atopy rates are higher in western countries with a high degree of industrialization than in developing countries with a large rural population (Kabesch, 2004). Living conditions on farms differ in many ways from those of other families: more pets, larger family size, heating with wood and coal, less maternal smoking, more dampness, and different dietary habits.

Mammalian TLR	Host defense / function	Selected natural ligands	Purified or artificial ligands	Signaling Pathways	Promotes or <b>inhibits</b> asthmatic inflammation <span>↑ ↓</span>
<b>TLR 1, 2, 6</b>	<b>Gram +ve bacteria, Mycobacteria, Yeast, Spirochetes</b>	<b>LTA, PGN, Lipoproteins, Zymosan, Glycolipids</b>	<b>Pam3Cys, Lipopeptides</b>	<b>MyD88, TIRAP</b>	<span>↑</span> <span>↓</span>
<b>TLR 3</b>	<b>Virus</b>	<b>dsRNA</b>	<b>Poly (I:C)</b>	<b>TRIF</b>	<b>?</b>
<b>TLR 4</b>	<b>Gram -ve bacteria</b>	<b>LPS, LTA</b>	<b>Lipid A</b>	<b>MyD88, TIRAP, TRAM, TRIF</b>	<span>↑</span> <span>↓</span>
<b>TLR 5</b>	<b>Flagellated bacteria</b>	<b>Flagellin</b>	<b>FliC</b>	<b>MyD88</b>	<span>↑</span>
<b>TLR 7</b>	<b>Virus</b>	<b>ssRNA in mice</b>	<b>Poly (U) Imiquimod R-484</b>	<b>MyD88</b>	<b>?</b>
<b>TLR 8</b>	<b>Virus</b>	<b>ssRNA in humans</b>	<b>Imiquimod</b>	<b>?</b>	<b>?</b>
<b>TLR 9</b>	<b>Bacteria and virus</b>	<b>CpG motifs in DNA</b>	<b>ISS</b>	<b>MyD88</b>	<span>↓</span>
<b>TLR10</b>	<b>?</b>	<b>?</b>	<b>?</b>	<b>?</b>	<b>?</b>
<b>TLR11</b>	<b>Uropathogenic</b>	<b>?</b>	<b>?</b>	<b>MyD88</b>	<b>?</b>

**Tab.1.1b Mammalian toll-like receptor ligands with known signaling pathways utilized by each receptor and their functional significance in asthma** (according to: Eisenbarth, 2004. Understanding asthma pathogenesis: linking innate and adaptive immunity. Cur.Opinion in Pediatrics, 16: 659-666)

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Therefore, the exposure to certain immune-modulators specific for farm life may prevent the occurrence of these conditions. Frequent contact with live stock is further associated with the protective effect of farm life. A dose-response relationship between exposure to farm animals and the prevalence of atopic disease was reported among farmer's children in Bavaria (Von Ehrenstein, 2000). More insights with regard to assumed microbial components responsible for the allergeo-protective effects came again from the farming studies suggesting that lipopolysaccharide (LPS), a Gram<sup>-</sup> bacterial membrane component might be one of these factors. The *Allergy and Endotoxin Study* (ALEX Study) shown that endotoxin concentration was highest in stables from farmer families and farmer house holds contain much more endotoxin that non-farmer environments. Indeed, it has become evident that immune responses against all those allergens are initiated by Toll-like receptors (TLR) signals that recognize a variety of structures derived from all kind of microorganisms (Tab.1.1b)(Bauer, 2007).

## 1.2 Prenatal physiological determinants and allergy development

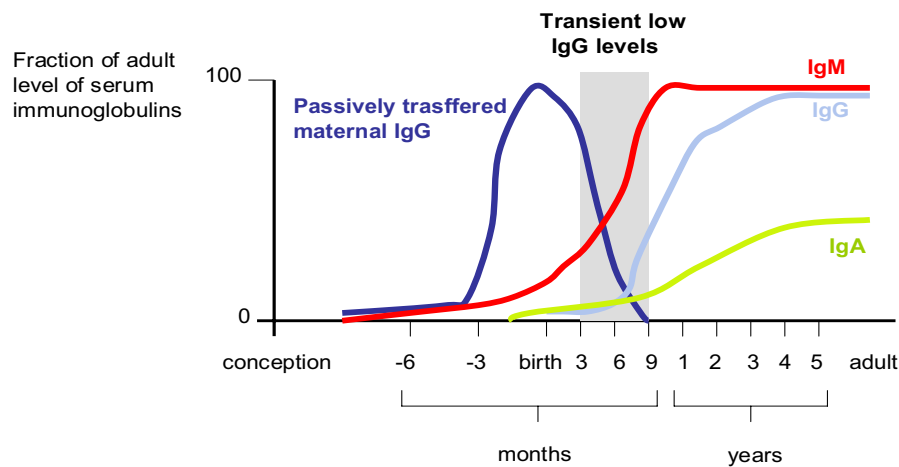
Pregnancy – a complex interaction between genetic, anatomic, endocrine, immune and neurological systems is a delicate balance of hormonal and immunological functions, almost certainly affected by environmental factors. The influence of the environment and other exogenous agents on mechanism of pregnancy plays an important role in the development of fetal and newborn immunity, and allergic diseases later in life. Increasing evidence suggests that prenatal and early postnatal environmental determinants play an important role in the development of allergy and asthma. Tolerance programming starts in early life, even before birth. Indeed, the presence of allergen specific T cells has been demonstrated in humans at the time of birth suggesting that specific immune responses are develop *in utero* (Prescott, 1999; Szepfalusi, 1997).

Prenatal events - have the capacity to contribute to airway inflammation on two broad levels: (i) through effects on the development airway structure and function, and (ii) by immune effects that may alter the risk of subsequent allergic

sensitization. The growth and development of the airway *in utero* appears to be an important determining factor in infant's lung function. Children with undeveloped airways are more likely to have milder respiratory tract symptoms with viral infections such as Respiratory Syncytial Virus (RSV) (Prescott, 2006; Martinez, 1995; Openshaw, 2003). Although, fetal immune responses are typically skewed towards  $T_H2$  in pregnancy (Prescott, 1998), a number of subtle differences have been found in neonates who go on to develop allergic disease (Prescott, 2003) and even if this was thought to be genetically programmed, there is now evidence that *in utero* exposures can influence fetal immune function. Maturation of the fetal immune system occurs primarily during the first two trimesters of pregnancy. The development of clinical tolerance continues after birth and the first two years of life seems to be particularly important (Prescott, 1998). Moreover, transplacental allergen transfer has been demonstrated in animals and humans (Holloway, 2000). The intrauterine allergen exposure may happen both, via transfer of allergens in the amniotic fluid - across fetal membranes and via different crossways of placental tissues. However, so far is not clear: (i) whether transplacental allergen transfer happens in general, (ii) at what stage of pregnancy it occurs predominantly and (iii) which transfer mechanism illustrates better the transplacental allergen passage (Loibichler, 2002). Nevertheless, the placental barrier plays an active role in the decision of whether an antigen crosses the placenta or not. In that way, the transplacental transfer of allergens may be part of a mechanism protecting the fetus. Before birth, professional antigen-presenting cells (APCs) in the peripheral blood, dendritic cells (DCs) are not equipped with a mature set of costimulatory molecules enabling them to present professionally antigen to circulating antigen-specific T cells. Such stimulation of T cells would lead to tolerance induction to those antigens encountered before birth (Szepfalusi, 2000; Szepfalusi, Loibichler, 2000). Physiological data indicate that, there are two pathways for allergen transfer across human placenta: transcellular and paracellular pathways (Sibley, 1988; Schneider, 1991). The two cell layers forming the exchange barrier are the fetal endothelium and the syncytiotrophoblast. Contrary to the endothelium, the

syncytium has no lateral cell membranes forming an intercellular space (Leach, 1992). However, the continuous structure of the syncytiotrophoblast is interrupted by degeneration. The gap is immediately filled by fibrinoid account for approximately 7% of the villous surface of the normal human term placenta (Nelson, 1990). Recently, the permeability for macromolecules through the syncytiotrophoblast discontinuities on placental villi has been reported for horseradish peroxidase (HRP) and alfafetoprotein ( $\alpha$ -FP) both with molecular weight of 40kDa (Edwards, 1993; Brownbill, 1995). Alternatively, the syncytium can be passed transcellularly. It is well known that, immunoglobuline G (IgG) crosses the placenta via the neonatal Fc receptor FcRn expressed on syncytiotrophoblast and endothelial cells (Saji, 1999; Leach, 1996; Simister, 1996) from early gestation, becoming maximal in the last week of pregnancy (Malek, 1996). The newborn has initial antibody levels comparable to those of the mother, because of the transplacental transport of maternal IgG. As the transferred IgG is catabolized, antibody levels gradually decrease until the baby himself begins to produce necessary amounts of IgG at about 6 months of age. Thus, IgG levels are quite low between the ages of 3 months and 1 year when active IgG antibody responses are poor. In some infants this can lead to a period of increased susceptibility to infection (Fig.1.2). Besides, lack of immunoglobulin A (IgA) can be associated with the predisposition to lung infections with various pathogens and is consistent with the role of IgA in defense at the body's surface. Based on those findings, we believe that controlled maternal allergen exposure could actively induce allergen-specific tolerance in the fetus. Potentially allergens encountered by the mother induce the transamniotic transfer of maternal IgE, such as gut-associated IgE receptor-mediated antigen, and consequently the allergic sensitization (Jones, 1999). Despite high molecular weights (MW> 500 kDa) of the other studied allergens it could be speculated that transplacental allergen transfer mainly occurs by a paracellular route (Tab. 1.2).





**Fig.1.2 Immunoglobulin levels in newborn infants fall to low levels at about 6 months of age.** Human babies are born with high levels of IgG, which is actively transported across the placenta from the mother during gestation. After birth, the production of IgA and IgM starts almost immediately, but the IgG levels delay between the ages of 3 - 9 months to 1 year of age can lead to susceptibility to allergic disease (according to: Janeway, CA. jr., 2006. Immunobiology - the immune system in health and disease, Handbook the 6<sup>th</sup> Edition).

Immunoglobulin									
	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
Heavy chain	$\gamma_1$	$\gamma_2$	$\gamma_3$	$\gamma_4$	$\mu$	$\alpha_1$	$\alpha_2$	$\delta$	$\epsilon$
Molecular weight (kDa)	146	146	165	146	970	160	160	184	188
Serum level (mean adult mg/ml)	9	3	1	0.5	1.5	3.0	0.5	0.03	5x10 <sup>(-5)</sup>
Half-life in serum (days)	21	20	7	21	10	6	6	3	2
Classical pathway of complement activation	++	+	+++	-	+++	-	-	-	-
Alternative pathway of complement activation	-	-	-	-	-	+	-	-	-
Placental transfer	+++	+	++	- +	-	-	-	-	-
Binding to macrophage and phagocyte FcRs	+	-	+	- +	-	+	+	-	+
High-affinity binding to mast cells and Ba	-	-	-	-	-	-	-	-	+++
Reactivity with Staphylococc. Protein A	+	+	- +	+	-	-	-	-	-

**Tab.1.2 The immunoglobulin isotypes.** There is evidence that exposure to allergen complexes with the active transport of IgG across the placenta mostly in the third trimester of pregnancy (according to: Janeway jr., 2006. Immunobiology - the immune system in health and disease, Handbook the 6<sup>th</sup> Edition).

In early postnatal period, environmental exposure plays a crucial role in driving immune maturation, which appears to depend on exogenous factors (microbial exposure) to develop normally. The pattern of environmental antigen exposure determines the specificity of responses required for host defense and local conditions during antigen-processing in local tissue appear to influence the patterns of immune maturation and resulting immune responses. Local encounters with noxious environmental factors, including irritants and respiratory pathogens, are likely to influence the development of immune networks in the airways and the propensity for chronic inflammation. In other words, it is now well recognized that natural exposure to microbes through mucosal surfaces in the gastrointestinal tract, respiratory tract and skin are critical for the development of clinical tolerance. As is mentioned, all these observations are directly linked to the *Hygiene hypothesis* which states that exposure to microbial agents play an important role in immunoprotection. Indeed, microbes are now viewed as important immunoregulators in addition to their role as pathogens (Renz, 2002). In conclusion, maternal and fetal allergen exposure is a common phenomenon, which challenges the conventional opinion that the unborn should be protected from pathogenic allergens but, the presence of allergens *per se* in fetal circulation may in fact be part of the normal development of immunity (Loibichler, 2002). As pregnancy is a  $T_H2$  - biased phenomenon, but allergy is characterized by a  $T_H2$  dominance too, the required postnatal  $T_H1$  immunity development could be influenced by the pregnancy  $T_H1/T_H2$  immune balance through antigen specific exposure, dose- and time-depending (Raghupathy, 1997; Wegmann., 1993).

### 1.2.1 Immunological factors

The immunological interaction between mother and the fetus remains a scientific mystery. In normal pregnancies, the maternal immune system does not react to spermatozoa or the embryo, even though they express antigens that are exogenous to the maternal system. Maternal-fetal tolerance has been compared

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to that of the semi-allogenic fetal graft and may be the result of a complex array of mechanisms, including: HLA-G expression of trophoblast, the leukemia inhibitory factor (LIF) and its receptor (LIFR), the indoleamine 2, 3-dioxygenase (IDO), the  $T_H1/T_H2$  balance, suppressive macrophages, hormones such progesterone (P), placental growth hormone (PGH), CD95 and its ligand, and annexin II), definitively are pregnancy-specific and interconnected (Thellin, 2003; Thellin, 2000). Immune responses can be triggered by a variety of endogenous and exogenous factors, as well as the production of anti-paternal antibodies, autoimmune disorders leading to the production of autoimmune antibodies (anti-phospholipid antibodies, anti-nuclear antibodies, and polyclonal B cell activation), infections, stress, and toxic agents (Thellin, 2003; Giacomucci, 1994).

### 1.2.2 Endocrine factors

Progesterone is one of the two main hormones (the other one being estrogen) produced each month by the ovaries of menstruating women (being produced in smaller amounts by the adrenals). It is the major female reproductive hormone during the later two weeks of the menstrual cycle, made by the corpus luteum of the ovary. It is normal for the levels of progesterone to rise and fall during the monthly cycle. Progesterone production starts just before ovulation each month and increases rapidly after ovulation. It prepares the lining of the uterus for the fertilized egg and maintains pregnancy. The name itself tells the story: *progesterone = promotes gestation hormone*. It acts on the reproductive tract in preparation for the initiation and maintenance of pregnancy by inhibiting contraction of the uterus and the development of new follicles (Niswender, 2000). Follow oocyte fertilization the developing embryo secretes human chorionic gonadotropin hormone (hCGH) which sustains progesterone levels. During pregnancy, fetoplacental estrogen, progesterone and adrenocorticoid hormones are secreted into both fetal and maternal circulation (Gabbe, 2002). It is produced by the placenta as well during pregnancy and in small amounts by the adrenal cortex. Moreover, progesterone maintains a normal menstrual cycle and

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plays an important role during pregnancy by preparing the tissues lining the uterus for implantation by the fertilized egg. It helps prepare the breasts for milk production. For mothers, progesterone plays a protective role in preventing some chronically diseases such as breast- and uterine cancer. It also stimulates normal bone growth and thyroid function. It has many opposite, balancing activities to those of estrogen. In addition to normalizing blood sugar levels and water metabolism, progesterone has in general a calming effect on the central nervous system too. Estrogen production is mainly under the control of the fetus and is primary signal by which the fetus directs essential physiologic processes that affect fetal well-being. By 20<sup>th</sup> week of pregnancy, approximately 90% of maternal estriol excretion can be accounted for by dehydroepiandrosterone sulphate (DHEA-S) production by the fetal adrenal gland. Estrogen affect progesterone production, uterine blood flow, mammary gland development and fetal adrenal gland function (Speroff, 1999).

### 1.2.3 Physical and chemical agents

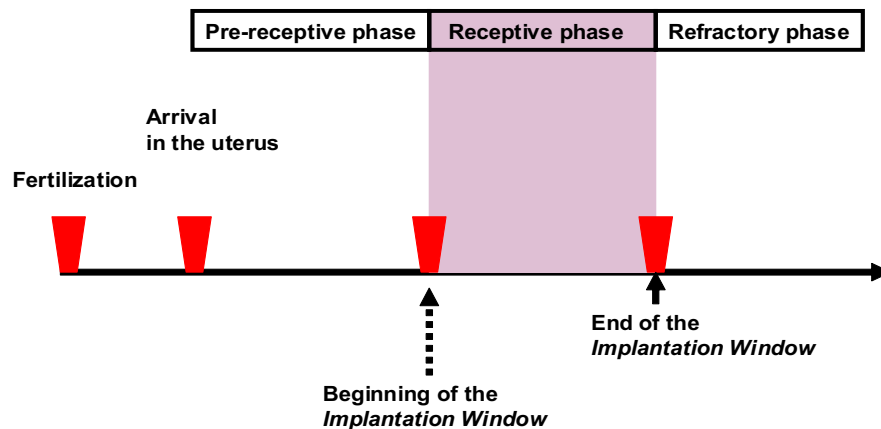
Immunotoxicity may occur when the immune system acts as a passive target to chemicals or it responds to the antigenic properties of a chemical as part of a specific immune response (Damstra, 2002). Toxic substances that alter metabolism or vascularization can disturb placental development and thus impeding or blocking mechanisms of tolerance and protection, and increase the chance of fetal rejection (Thellin, 2003).

## 1.3 Fetal development of innate and adaptive immunity

### 1.3.1 The implantation window

Implantation is different from the *established pregnancy* in that it involves intensive tissue remodeling to create a new foeto-maternal organ, placenta. Thus, *de novo* created new organ evolving from trophoectoderm derived from the invasive ectoplacental cone to create the differentiated placenta (Chaouat, 2006).

The uterus and the blastocyst must be synchronized to allow suitable implantation and development. Synchronization results from the adjustment of the blastocyst development and the status of the endometrial receptivity. In the majority of species, the implantation takes about 1-2 days and if asynchrony exceeds, this interval of time implantation is severely impaired. However, the uterus must be ahead of the embryo in terms of cycle status. The embryo is able to adapt to a poorly synchronized uterine environment by arresting its development. An extreme example of that blastocyst capacity is given by species presenting delayed implantation. The uterine differentiation is less flexible and follows the pre-programmed evolution driven by the steroids hormones during the cycle (Fig.1.3.1).



**Fig.1.3.1 Schematic illustration of the implantation window** – pre-receptive, receptive and refractory phases occur in pregnant uterus during implantation (according to: Chavatte-Palmer P. and Guillomot M., 2006. Comparative implantation and placentation, Embryo implantation: from basic to clinics. Handbook of 1<sup>st</sup> EMBIC Summer School)

During the *pre-receptive* and *receptive* phases the embryonic development is sustained by a favorable uterine environment, whereas at the *refractory* phase, the uterine environment becomes hostile for the embryo. Perhaps, the uterine *refractory status* depends on the synthesis and secretion of embryotoxic factors in the uterine environment. The timing range of the *implantation window* is species specific. It needs that the uterus undergoes specific tissue remodeling, a process which is characterized by four important events: (i) decidualisation,

which involves an important change of the uterine stroma and uterine adhesion molecules; (ii) transformation of the uterine arteries into functional spiral arteries; (iii) several cellular fluxes; (iv) transient secretion of inflammatory cytokines. Thus, the implantation process initiates when embryos at the blastocyst stage breach the uterine epithelial cell layer, endometrium, creating the beginning of the intimate interactions between the zygote and maternal systems. At this time, the pregnant uterus undergoes a radical structural transformation. Decidualization, the proliferation and differentiation of uterine stromal cells, increases the thickness of the uterine wall by approximately five fold. Endometrial stromal cells, at the site of implantation begin to differentiate into primary decidual cells. Cells that are more distal to the implantation site differentiate into secondary decidual cells (Theiler, 1972; Theiler, 1983). This differentiation and reorganization of cells allows the placenta to be positioned in the mesometrial decidua (Hunt, 1997). Uterine NK (uNKs) cells traffic to the site of implantation, proliferate immediately and become mesometrial localized. Here uNK cells form a lymphocyte-rich structure known as the metrial gland or *mesometrial lymphoid aggregate of pregnancy* (Adamson, 2002; Ashkar, 2000). They are a major source of interferone gamma (IFN $\gamma$ ) (Platt, 1998; Saito, 1993), which plays a key role in early pregnancy, inducing decidual artery remodeling (Ashkar, 2000). IFN $\gamma$  has been shown to regulate  $\alpha_2$ -macroglobulin, a regulator of proteases and cytokines produced from the mesometrial decidua that are important for successful pregnancies. But, a vast subpopulation of NKT cells has been observed in the peri-implantation uterus. These cells express the NK marker NK1.1 and are V $\alpha$ 14<sup>+</sup> and CD4<sup>-</sup>/CD8<sup>-</sup>. In mice, these cells are generally restricted by the non-classical MHC molecule CD1d, express TCR $\alpha$  chains with invariant usage of V $\alpha$ 14 and J $\alpha$ 281 genes, and recognize a variety of non-mammalian glycolipids, like  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), (Matsuda, 2001). V $\alpha$ 14 NKT cells are suspected to react with an unidentified fetal antigen that appears to regulate their expansion (Dang, 2001). Neutrophils are almost exclusively limited to the leading edge of enzymatic digestion. At this site they are likely involved in

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phagocytosis of cellular waste from decidual cells killed by invading trophoblasts (Parr, 1990).

### 1.3.2 Adhesion - Embryo division and maturation

To implant, the embryo itself has to undergo several stages: the initial divisions first depends on the expression of Organic Cation Transporter 3 transcription factor (OCT3), expressed in germ cells, blastomers until morula stages, and later divisions are controlled by the mitosis promotion factor, an heterodimer of the phosphor protein cell division cycle 2 (Cdc2) and cyclin B. After a variable period of time in the uterine horn, the free floating embryo positions itself near the uterine epithelium (apposition stage) and then adheres by both mechanical and chemical junctions or bonds. The first steps of this process are very similar to the ones seen in the rolling and adhesion events seen when a lymphocyte homes or adheres to a vessel. In this process, an important role is played by a series of complementary adhesion molecules, such as E cadherins and integrines. Early human blastocysts express  $\beta 1$  and  $\beta 5$  integrin subunits, E cadherin and zonula occludens 1 protein (ZO-1). In both cases of adhesion of a lymphocyte to the endothelial wall and of adhesion of the embryo, a key role is played by L-selectin, and antibodies to L-selectin do block implantation, (Genbacev, 2003). The expression of adhesion molecules is similar to an inflammation process, with high local expression of such inflammatory molecules as interleukin 1 (IL-1), colony-stimulating factor 1 (CSF-1), interferone gamma (IFN $\gamma$ ) and tumor necrosis factor alfa (TNF $\alpha$ ). In variance with what is seen in established pregnancy, several of these inflammatory cytokines are necessary for successful implantation. The IL-1 system has been implied by C.Simon et al. as mandatory, since IL-1 RA injection was claimed to prevent implantation (Simon, 1997). Two cytokines seem very important: IL-11, which plays a role in the formation of the decidual tissue at implantation site. IL-11R KO mice are sterile (Bilinski, 1998) and in human a subset of sterile women have too low uterine IL-11 production. LIF was the first cytokine shown by KO to be obligatory for implantation: LIF KO mice do not

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implant, even in case of transfer of LIF<sup>+</sup> producing embryos and this defect is corrected by maternal infusion with recombinant LIF (Stewart, 1992). In human, too low level of LIF induce sterility (Delage, 1995; Laird, 1997), but too high level seems to reflect a *chronic T<sub>H</sub>1* - like syndrome, and thus also correlates with sterility. Another important cytokine at that stage is indeed CSF-1, which acts as a uterine growth factor for trophoectoderm and later on differentiated trophoblasts (Bartocci, 1986).

### 1.3.3 Embryo – *the wonder graft*?

After adhesion, embryo becomes a *graft*. Before adhesion he required the hatching from the zona pellucida, and prepared by embryonic signals. Until hatching, the zona pellucida has protected the embryo from immune attack and immune recognition. Adhesion and invasion will confront the outer layers of the embryo with the maternal immune system. At this stage, a broad and increasing number of uNK cells occur, but relatively few local T cells and most of the macrophages which were present in the early steps have been missing from the future decidua basalis, a phenomenon termed by Robert Fauve *immuno – repulsion*. The few T cells which remain are mostly but not exclusively,  $\gamma\delta$ T cells. That the T cells do recognize the foetus is exemplified by the fact that maternal antipaternal alloantibodies are detectable in pregnant women (Bell, 1983). To minimize any risk of pregnancy, the placenta has a selective and programmed expression of major histocompatibility complex (MHC) antigens. In mammals, there never is expression of MHC class II antigens on placenta (in mice cause abortion and implantation failure). There is expression in rodents of polymorphic MHC antigens (H-2 K, D and L in mice) on the outer layers of the placenta, the spongiotrophoblast (Zuckermann, 1986). The human placenta seems to evolve towards a polymorphic MHC negative barrier, the villous syncytiotrophoblast being MHC - class I negative at least as far as membrane expression is concerned, whereas the so-called extravillous trophoblast has lost expression of HLA-A and HLA-B, but maintains expression of HLA-C (King, 2000; Le Boutellier,



2000). From immunological point of view there are many threats to well development of pregnancy, such as alloantibodies, complement system, CD8<sup>+</sup>T cells or NKs. In fact, there is a specific protection to counter it is exemplified by data from Xu et al., where KO mice for complement receptor 1-related gene/protein y (CRRY) results in early peri-implantation fetal destruction in both syngeneic and allogeneic breeding (Xu, 2000). The equivalent protection in human and mice is offered by membrane cofactor protein (MCP) and decay accelerating factor (DAF), and it is probable the cause, that a part of early embryonic losses in human are related to lack of expression of such factors. The role of complement, once its cytotoxic effects regulated the placental growth. Another risk results from activated CD4<sup>+</sup>T cells, which induce the neutralization activity of indoleamine 2, 3-dioxygenase (IDO), that causes abortion in allogeneic but not syngeneic pregnancy (Mellor, 2004). IDO might be also involved in the generation of paternal antigen specific T suppressor cells and T<sub>regs</sub> which have been implied in the success of pregnancy (Chaouat, 1980; Aluvihare, 2005). The most important risk to the fetus is caused by NKs. Also, there is obviously that up-regulation of NK cytotoxic activity induces implantation failure or early pregnancy loss by CD8<sup>+</sup> differentiated cytotoxic T cells in mice, too (Baines, 1993). It is important to mention that NK cells are continuously inhibited in physiological conditions by inhibitory killer Ig-like receptors (iKIRs) binding to self MHC. The absence of the proper self MHC at membrane surface of a cell prevents such a binding, diminishing the inhibition process, and lysing the target cell. But the cells also display an array of activator KIRs receptors and ligation of these by MHC class I molecules induces the secretion of cytokines (Lanier, 2005; Parham, 2004; Dietl, 2006). A significant role is also played by decidual dendritic cells (DCs). Some of their cytokines are required for successful pregnancy (Genbacev, 2003).

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### 1.3.4 The invasion process accompanied by uterine modifications

The invasion process represents the first step towards the establishment of what Peter Medawar in 1953 defined as *the Nature's allograft*, where a minimal immunosuppression is needed. The invasion process involves penetration of the stroma after degradation of extracellular stromal matrix. Invasion requires a balance of adhesion molecules and under hormonal condition of embryonic matrix metalloproteases (MMPs) and tissue inhibitors of MMP (TIMPs), with a net balance in favor of proteases, such as: MMP above TIMPs and urokinase plasminogen activator (uPA) above plasminogen activator inhibitor-2 (placental PAI-2). In fact, LIF is very important in the initiation of implantation by activation both proteases and protease inhibitors, but the final net balance is in favor of proteases. In general, these molecules are themselves under the up- and down-regulation inflammatory and anti-inflammatory cytokines, which exert opposite effects. IL-1 $\beta$  activates MMPs, inhibits TIMPs, whereas TGF- $\beta$  down regulates it. TGF- $\beta$  is found in great amount in the decidua basalis. Consequently, the embryo promotes its invasion, whereas the uterus resist to it. However, the blastocyst implantation implies profound changes of the endometrium, which will favor adhesion of the trophoblast and limit it to the proper location in the uterus. Major cellular changes of the endometrium involve both the luminal epithelium and the stroma. In rodents and human the epithelial cells transform as bulbous structures named pinopodes during the luteal phase of the cycle which corresponds to the *implantation window* (Given, 1989; Nikas, 1999). In rodents, the transformation may be associated with reabsorption (endocytosis) of the luminal fluid by the epithelial cells. The stromal response to implantation is diversely pronounced according to species but in most species, inflammatory manifestations are observed such as an edema associated with increased of capillary permeability and vasculogenesis. The most pronounced stromal transformation is the decidual reaction observed in species with invasive implantation. The decidualisation is complete in rodents and primates. In rodents, the decidual reaction is localized to the implantation site, whereas in higher

primates or human, the whole endometrium is affected. In these species, a similar reaction is observed at the end of the cycle and is followed by the menses. From the cytological aspect, it is an epithelial differentiation of the stromal fibroblasts associated with a proliferation and polyploidy. The decidual reaction is triggered by the blastocyst at time of implantation. Uterine decidualisation can be induced by various inflammatory stimuli: physical (trauma), chemical (CO<sub>2</sub>), and physiological (prostaglandins PGF $\alpha$ -2 and PGE<sub>2</sub>, histamine, platelet-activating factor PAF). But all the other factors are effective on ovarian steroids, estradiol (E<sub>2</sub>) and progesterone (P<sub>4</sub>), where the ratio E<sub>2</sub>/P<sub>4</sub> in pregnant endometrium became optimal only with an intact luminal epithelium. The decidual cells produce glycogen, alkaline phosphatase (AP),  $\alpha$ -Actin, prolactin and growth factors such as transforming growth factor -alfa and -beta (TGF- $\alpha$  and TGF- $\beta$ ), insuline like-growth factor (IGF) and insuline like-growth factor binding protein-1 (IGF-BP-1), tumor necrosis factor-alfa (TNF- $\alpha$ ), granulocyte-macrophage colony-stimulating factor (GMC-SF), which play a role in trophoblast growth and differentiation.

### 1.3.5 Hormonal influence upon pregnancy cytokine pattern

Possible candidates include the measurement of various molecules involved in implantation, sampled by uterine flushing or endometrial biopsy. Mediators like as metalloproteases, integrins and cytokines have been successfully quantified. Cytokines are among the most of those mediators. In all aspects, a common sequence of hormonal impregnation is needed for preparation of the endometrium in implantation. Ovarian steroids, estradiol (E<sub>2</sub>) and progesterone (P<sub>4</sub>) produced during the oestrous cycle are the main factors which control the endometrial receptivity. The pre-ovulatory surge of E<sub>2</sub> acts on proliferation and differentiation of the uterine cells. Progesterone stimulates the synthesis of uterine secretions which sustain the embryonic development until it implants, but progesterone receptors disappear from uterine epithelium just prior to implantation in most species (Spencer, 2004). According to the species at time of

implantation, E<sub>2</sub> stimulation is necessary in rodents but not in human. Various paracrine factors like histamine, platelet activating factor (PAF), prostaglandins and pro-inflammatory cytokines such as colony stimulating factor-1 (CSF-1), interleukins (IL-1, IL-2, IL-3, IL-11), transforming growth factor beta (TGF- $\beta$ ) and leukemia inhibitory factor (LIF) are produced by the receptive endometrium under the control of P<sub>4</sub> and/or E<sub>2</sub> (Carson, 2000). Receptors of these cytokines have been found on the blastocyst. Thus it is likely that implantation is under the control of complex exchanges between the uterus and the embryo, but most of these mechanisms remain to be determined. Also, the steroid hormones may initiate a downstream cascade of molecular events through local paracrine/autocrine molecules. The attention is focused on cytokines that have been documented in KO mice model as essential for the implantation process.

In 1992, Stewart *et al.* demonstrated the absolute necessity of leukemia inhibitory factor (LIF) in murine implantation by monitoring the reproductive performance of LIF KO mice (Stewart, 1992). The implantation blockade observed in LIF-mice could be partly corrected by an intra-peritoneal administration of recombinant murine LIF (Chen, 1995). LIF protein and mRNA are present in the human endometrium throughout the cycle but increase significantly during the luteal phase (Vogiagis, 1996; Arici, 1995; Charnock-Jones, 1994). The human endometrium epithelial cells express mRNA for LIF receptor components as well as the blastocyst itself (Cullinan, 1996; Chen, 2000). Those findings suggest that LIF plays an important role in the embryo-endometrium communication (Haines, 2000). The presence of other cytokines was detected in human endometrium such as CSF-1, TNF or IFN families, IL-11, IL-8 and the remarkable CD56<sup>+</sup> cells so-called uterine natural killer cells (uNKs).

Many observations suggest that pregnancy is associated with an altered T<sub>H</sub>1/T<sub>H</sub>2 balance and thus, normal pregnancy is characterized by a T<sub>H</sub>2-biased cytokines balance at the periphery. Also, immunoglobulin synthesis in pregnant women is increased (Myers, 1985), whereas cell-mediated responses are decreased (Santoli, 1976). The incidence of viral infections (Pickard, 1968) and tumors (Gleicher, 1979) is higher in pregnant women than in non-pregnant individuals.

Cytokines relevant to pregnancy may be generally divided into two categories, some of them being harmful for pregnancy by increasing cell-mediated immunity ( $T_H1$  type cytokines) and others exerting a beneficial effect by inhibiting strong cellular responses ( $T_H2$  type cytokines). The ratio of  $T_H1/T_H2$  type cytokines is significantly higher in supernatants of decidual cells, than in those peripheral lymphocytes (Wegmann, 1993). Low doses of GM-CSF, IL-3 or anti-TNF $\alpha$  reduce resorption rates in a murine abortion model (Chaouat, 1989), whereas administration of TNF- $\alpha$ , IFN- $\gamma$  or IL-2 to normal pregnant mice causes abortions (Parant, 1990). But, the picture is more complex at the feto-maternal interface, where cytokines are not constantly secreted by immune cells (Chaouat, 2004). Inflammatory cytokines like LIF are needed for implantation, whereas IFN- $\gamma$  is needed at low quantity for vascular development and transformation of uterine spiral arteries. The production of these cytokines is influenced exclusively by hormonal environment. LIF production (essential for embryo implantation) is up-regulated by IL-4 and progesterone, and is down-regulated by IL-2, IFN- $\gamma$  and IFN- $\alpha$  (Piccinni, 1998). Antigen-specific T cell clones derived in the presence of progesterone, exhibit significantly increased ability to produce IL-4 and IL-5 in comparison with T cell lines derived in absence of progesterone, and antigen-specific T cell lines generated in the presence of P developed into T cell clones showing a  $T_H0$  instead of  $T_H1$ -like cytokine profile (Piccinni, 1995). In vitro PIBF treatment of activated lymphocytes favors the production of  $T_H2$  type of cytokines (Par, 2000). Joachim et al., detected reduced PIBF concentrations, together with increased resorption rates where corrected by treating the animals with progesterone, and this was accompanied by a significantly increased decidual IL-4 production. Signal-transducing activators of transcription (STAT) such proteins like STAT6 and STAT4 specifically mediate signals from IL-4 and IL-12 receptors, respectively (Nelms, 1999). PIBF induces phosphorylation as well as nuclear translocation of STAT6, and exerts a negative effect on STAT4 phosphorylation. Thus, during normal pregnancy the PIBF demonstrated immunomodulatory effects of PIBF, mediated via altered cytokine production of the lymphocytes. Furthermore, the altered  $T_H1/T_H2$  balance observed in pregnant

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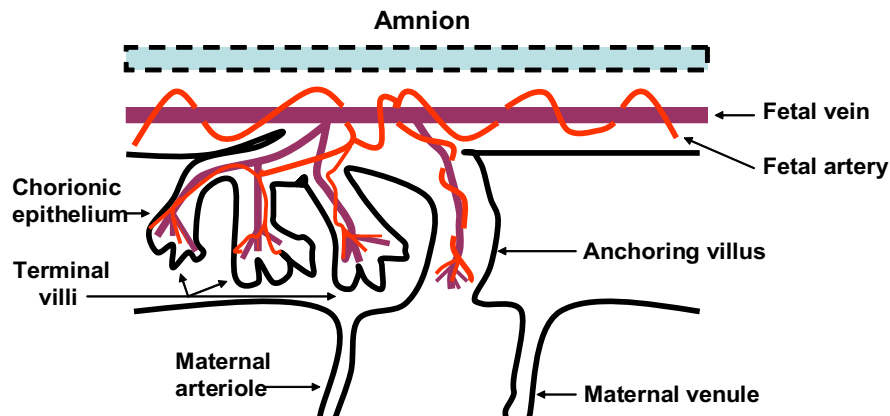
individuals might be attributable to the described effect of PIBF. The fetal-placental unit is a semi-allograft because of the paternal genetic contribution. Subsequently, there is a maternal immune response to the allogeneic pregnancy. The constituents of the maternal immune reaction to the allogeneic stimulus are not different from any other immune reaction and allogeneic conceptus (trophoblast) is in principle like all other allogeneic tissue grafts. The immunologic recognition of pregnancy and the subsequent activation of the maternal immune system is necessary for a successful pregnancy. It results in an upregulation of progesterone receptors on activated lymphocytes among placenta cells and decidual immune cells. Accordingly, the immunological pregnancy protective effects of progesterone might be manifested through the subsequent mechanism: after recognition of fetally derived antigens, activated lymphocytes develop progesterone receptors.

#### **1.4 Comparative Placentation: Human *versus* Mouse**

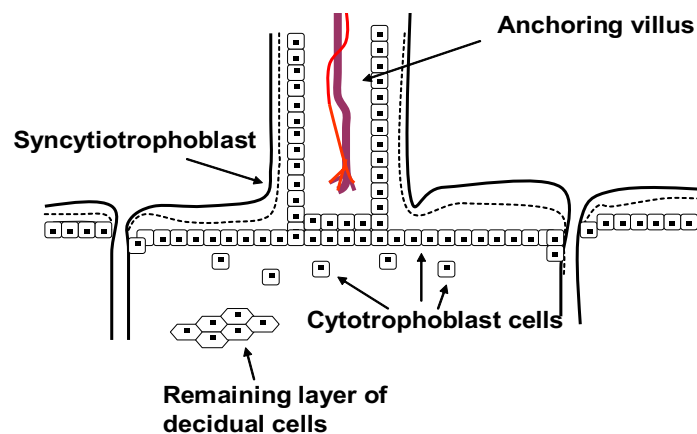
##### **1.4.1 Human placenta development and trophoblast differentiation**

The phenomenon of placentation passes through several phases before reaching its mature form found in term pregnancies, but the interfaces relevant to reproductive immunology and the basic structure of the placenta at term which is responsible for the physiological function of the placenta are completed quite rapidly. At the end of first trimester of pregnancy, the most important phase of maturation has been completed. This explains in part, the reason why invasive behaviour of trophoblast cells is detected mainly in the first trimester and is practically cancelled out at term. The primitive trophoblast (epithelial-like cell) of the chorion frondosum erodes the decidua, destroying glands and stroma but leaving the maternal arterioles and venules. These dilate to form sinusoids. The chorionic villi, lying in a pool of maternal blood, divide repeatedly to form complex tree-like structures in which branches of the umbilical vessels form vascular cascades closely related to the surface trophoblast epithelium. The branches of the original villi, themselves termed villi, are of two types (Fig.1.4.1a, b).

The majority float freely in the maternal blood and are known as terminal villi. A large number are attached to the maternal tissue forming the anchoring villi.

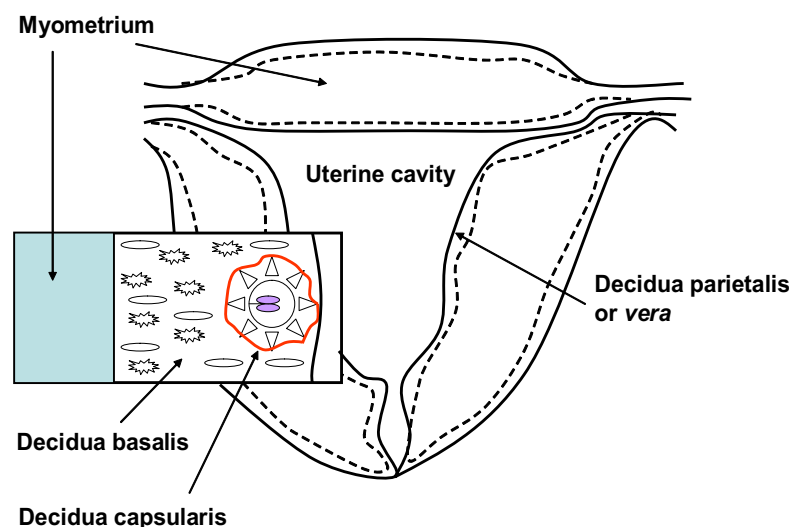


**Fig.1.4.1a Schematic illustration of the villi branches.** Fetal artery and vein invade the chorionic epithelium of terminal and anchoring villi. They are separated from maternal blood through different trophoblasts layers (according to: Miller A., Clander R., 1989. Obstetrics illustrated, Handbook, 4<sup>th</sup> Edition)



**Fig.1.4.1b The anchoring villus intimate architecture.** The inner cell layer forms Cytotrophoblast cell layer, while the outer cell layer the Syncytiotrophoblast cell layer (according to: Miller A., Clander R., 1989. Obstetrics illustrated, Handbook, 4<sup>th</sup> Edition)

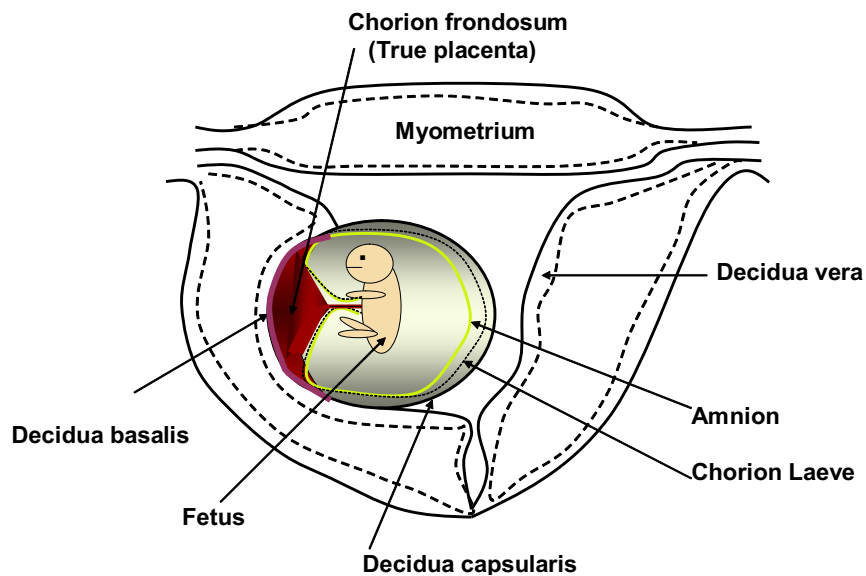
The trophoblastic epithelium lines the whole cavity containing the maternal blood in which the terminal villi are suspended. The syncytiotrophoblast possesses, among other properties, some of characteristics of vascular endothelium thus preventing the possibility of trombosis. Villi are present over the whole surface of the blastocyst. As the blastocyst enlarges it compresses the superficial decidua (decidua capsularis) and invades into the uterine cavity (Fig.1.4.1c).



**Fig.1.4.1c Implantation process in normal human pregnancy.** Three types of decidua are present during pregnancy evolution: decidua capsularis, decidua vera and decidua basalis (according to: Miller A., Clander R., 1989. Obstetrics illustrated, Handbook, 4<sup>th</sup> Edition)

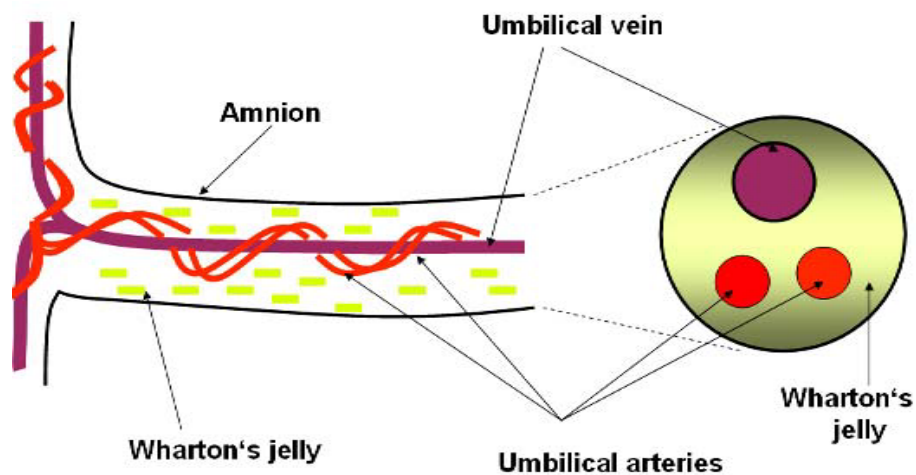
The compression of the decidua capsularis gradually cuts of the circulation through it. The result in atrophy and disappearance of the villi is in association with it. The surface of the blastocyst becomes smooth and this portion of the chorion is known as the chorion leave. At the opposite pole of the blastocyst the villi proliferate and enlarge and this is known as the *chorion frondosum*. The connecting stalk of the embryo is attached to the wall of the blastocyst at this point. Ultimately with the expansion of the blastocyst the *decidua capsularis* comes in contact with the *decidua vera* and the uterine cavity is obliterated (Fig.1.4.1d).





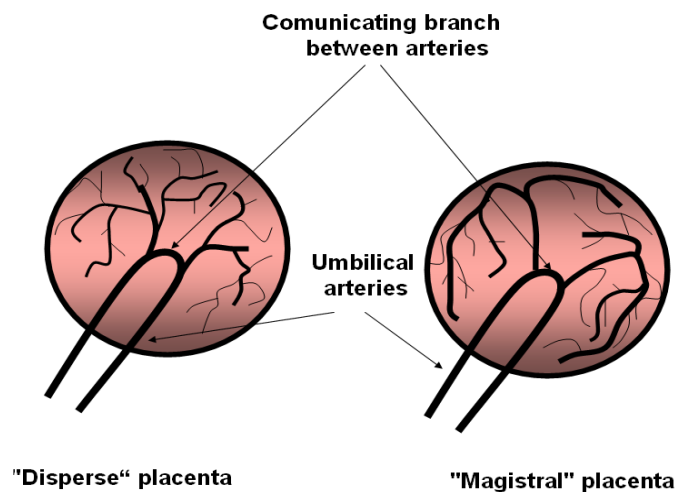
**Fig.1.4.1d Human fetal development.** The functional placenta develops in the same time with the foetus, providing him an authentic comfort into uterus (according to: Miller A., Cllander R., 1989. Obstetrics illustrated, Handbook, 4<sup>th</sup> Edition)

The umbilical cord has two arteries - one vein (human), and one artery - one vein (mouse), embedded in Wharton's jelly which is a loose myxomatous tissue of mesodermal origin. This jelly acts as a physical buffer and prevents twisting of the cord and interference with circulation (Fig.1.4.1e).



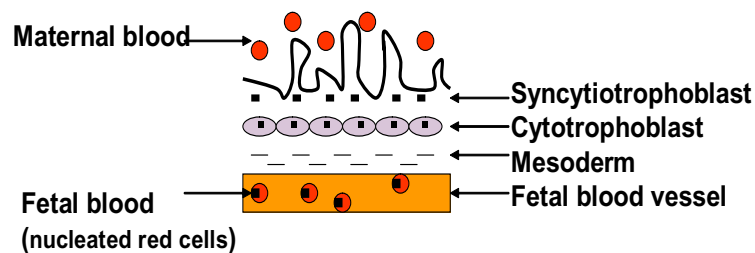
**Fig.1.4.1e Development of the human umbilical cord** The umbilical cord has two arteries-one vein in human, embedded in Wharton's jelly (according to: Miller A., Cllander R., 1989. Obstetrics illustrated, Handbook, 4<sup>th</sup> Edition)

The umbilical vessels are generally attached to the placenta near its centre. They immediately divide repeatedly to form branches all over the surface (*disperse placenta*) or sometimes, the main vessels may extend almost to the margins of the placenta before dividing (*magistral placenta*) (Fig.1.4.1f).



**Fig.1.4.1f Umbilical cord vessels dispersing human healthy term placenta** - there is a short communicating branch between the two umbilical arteries just as they reach the placenta surface. This serves to equalize the pressure and flow to each half of the placenta. (according to: Miller A., Cllander R., 1989. Obstetrics illustrated, Handbook, 4<sup>th</sup> Edition, 1989)

The fully formed human placenta is a disc, approximately 2.5 – 3.0 cm in thickness, tapering towards the edges. It weights roughly 500 g and is dark red, the color being due mainly to the maternal blood in the intervillous spaces. The functions of the placenta depend on the structure and health of the placental villi. These villi are bathed in maternal blood but there is not direct connection between fetal and maternal blood. The structures between the two circulations make up the so-called *placental barrier* (Fig.1.4.1g).



**Fig.1.4.1g Placental barrier.** Human and rodents present identical placental barrier architecture (according to: Miller A., Cllander R., 1989. Obstetrics illustrate, Handbook, 4<sup>th</sup> Edition)

The barrier effect is reduced in two ways. Small polyploidy extensions of the syncytial cytoplasm – microvilli - increase the surface for absorption, secretion and interchange between the two circulations. In human, as the pregnancy increases in size especially after the 16<sup>th</sup> week, the syncytiotrophoblast is reduced in thickness without alteration of its microvillous structure. The fetal mesoderm is reduced in amount and the vessels of the villi dilate. Thickness of placental barrier at 12<sup>th</sup> week is 0.025 mm, in comparison with term placenta 0.002 mm.

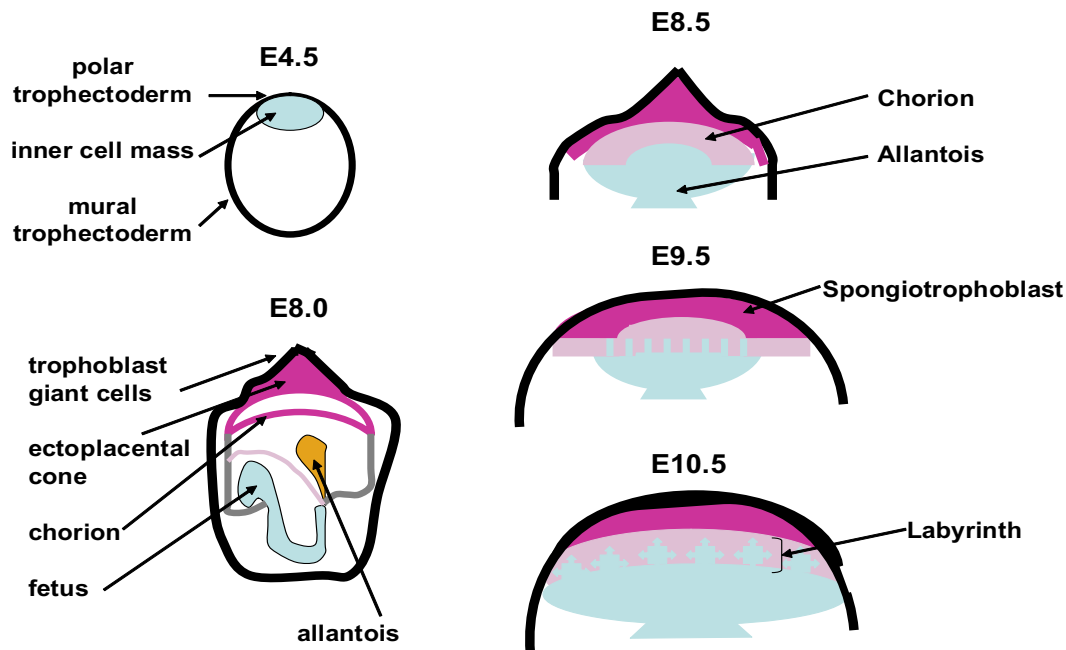
#### 1.4.2 Mouse placenta development and trophoblast differentiation

The mouse placenta is derived from multiple cell lineages being a combination of both fetal and maternal tissues. Trophoblasts, epithelial-like cells, derived from the trophoblasts lineage, invade the uterus to establish an intimate contact between fetus and mother (Cross, 2003). To control the maternal response to invasion, additional roles have been evolved in the mammalian placenta, including: (i) the production of hormones to adapt systemic maternal functions, and (ii) to provide a broad of growth factors to adjust the local uterine environment. But many of these functions are observed across different species evolution, too (Painter, 2002; Callard, 1993; Hughes, 1993; Renfree, 1993). In mice, twelve hours after fertilization on E0.5 (embryonic age ½ day, respectively 12 hours after conception), a sequence of cellular divisions create a cellular structure like-ball of undifferentiated cells termed *morula* (it is assumed that

fertilization occurs at the mid-point of the dark cycle, usually midnight, for mice kept on a 12 hours light ↔ 12 hours dark cycle). By E3.5 the blastocyst forms between 32 - 64 cells. Two different cell types can be distinguished at this time point: the inner cell mass (ICM) and trophoectoderm. The ICM consists of the innermost cells of the blastocyst. They are undifferentiated stem cells which give rise to the entire embryo proper as well as the mesenchymal (mesodermal) components of the placenta such as stromal cells and blood vessels. The ICM (inner cell mass) differentiates into the three germ layers of the embryo during gastrulation: (i) endoderm or hypoblast, (ii) mesoderm, and (iii) ectoderm or epiblast. Primitive endoderm begins to differentiate at approximately E4.0 from the free surface of the ICM of the blastocyst. This lineage has been demonstrated to only differentiate into the extraembryonic parietal and visceral endoderm of the yolk sac. Primitive endoderm does not contribute to the endodermal tissues of the fetus (Gardner, 1982). At approximately E4.5 the core ICM cells of the blastocyst derive the primitive ectoderm that divides rapidly and generates all tissues of the fetus itself (Theiler, 1972; Theiler, 1983). The cells outside of the blastocyst differentiate into an epithelium so-called trophoectoderm which contains the first cells of the trophoblasts lineage that will contribute exclusively to the epithelial composition of early placenta until term. The trophoectoderm cells that are adjacent to the ICM will become polar trophoblasts and those, not adjacent to the ICM will become mural trophoblasts. At the implantation time point, mural cells stop dividing, become large and form the primary trophoblasts – giant cells. Polar trophoectoderm cells remain diploid, maintaining proliferation and later will become polyploidy giant cells, respectively chorion cells generating an extensively placental tissue (Theiler, 1972; Theiler, 1983; Hogan, 1994). The labyrinth's morphogenesis begins with interposition of allantoic cells and fetal blood vessels into the chorionic plate. The labyrinth contains both trophoblasts and mesodermally derived cells from embryonic origin. Labyrinth growth can be separated into four distinct steps: (I) chorioallantoic fusion (attachment of the allantois to the chorionic plate) at E8.5, (II) early morphogenesis and syncytiotrophoblast differentiation, (III) expansion of

the labyrinth, and (IV) vascularization of the labyrinth (Cross, 2001). The labyrinth begins to function as a nutrient transport unit around E10.5, permitting the exchange of gases, nutrients and wastes between mouse mother and fetus. The closest region to fetus of the placenta is called - the chorionic plate. Here the yolk sac inserts and the umbilical cord originates. Fetal capillaries derived from the umbilical blood vessels branch from the chorionic plate and ending with a network of sinuses containing maternal blood in the labyrinth (Zuckermann, 1986). Finally, although the placental vascular system is not a distinct layer, it is considered one of the most critical features of the placenta, because a systematic description of placental functions would be incomplete without it. Blood vessel formation in the placenta occurs by angiogenesis and is regulated by multiple genes. Also, at E10.5 maternal blood vessels pass through the giant cell and spongiotrophoblast layers and reach the labyrinth zone forming close contact with fetal capillaries. The mature mouse placenta is established already by day E10.5. The zygote-derived components of the mature placenta consists of trophoblasts (extra embryonic ectoderm), endothelial and stromal cells (extraembryonic mesoderm), (Rodriguez, 2004). Within the placenta, the trophoblasts lineage has differentiated and reorganized to generate cell subtypes with distinct endocrine, vascular, immune and transport function capabilities (Cross, 2001). Moreover, by E13.5 of placental development, the spongiotrophoblast layer becomes adjacent to the maternal component of the placenta as the giant cells by this point are no longer maintained (Jaffe, 1990). Thus, an abnormal development or function of labyrinth can result in impaired fetal development (Rodriguez, 2004), (Fig.1.4.2a).

Similar to human placenta, the mouse placenta is named “haemochorial” (maternal blood vessels are in direct contact with placenta trophoblasts), (Wooding, 1994). Further development of the placenta relies on the interdependence of allantoic mesodermal cells and chorion trophoblasts (Hanato, 2003).



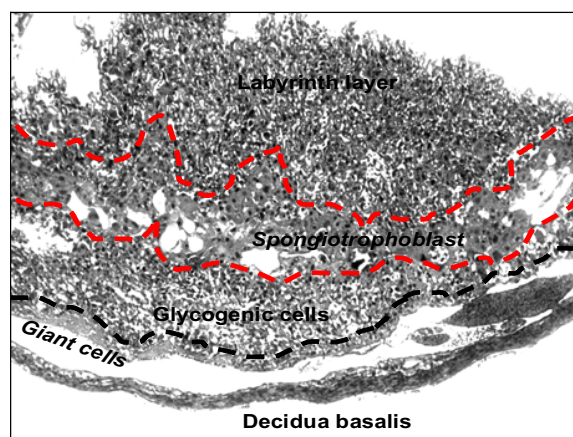
**Fig. 1.4.2a An overview of placental development in mice – morphological aspects of early placental development** (according to: Cross JC., 2005. How to make a placenta: Mechanisms of Trophoblast Cell Differentiation in Mice – A Review. IPFA 2004 Award in Placentology Lecture. Placenta, vol 26, suppl A, Trophoblast Research, vol 19)

The fully developed placenta contains four main layers: (i) the giant cell layer, (ii) the spongiotrophoblast layer so-called *junctional zone* and few glycogen trophoblasts cells (forming together the *feto-maternal interface*), (iii) the labyrinth layer (*placental barrier*), and (iv) the chorionic plate (contains *trophoblast stem cell layer* until pregnancy term).

The trophoblasts giant cells form the outmost layer of the placenta, are directly adjacent to maternal tissues, and perform several functions. They are a very invasive cell being the first to mediate implantation and the process of migration into the uterine wall (Cross, 2001). Later they produce several hormones and cytokines that promote both local and systemic physiological adaptations in the mother, including the regulation of maternal blood flow to the implantation site, production of progesterone from the ovary, lactogenesis and pancreatic islet hyperplasia (Linzer, 1999; Cross, 2002). The majority of primary and secondary

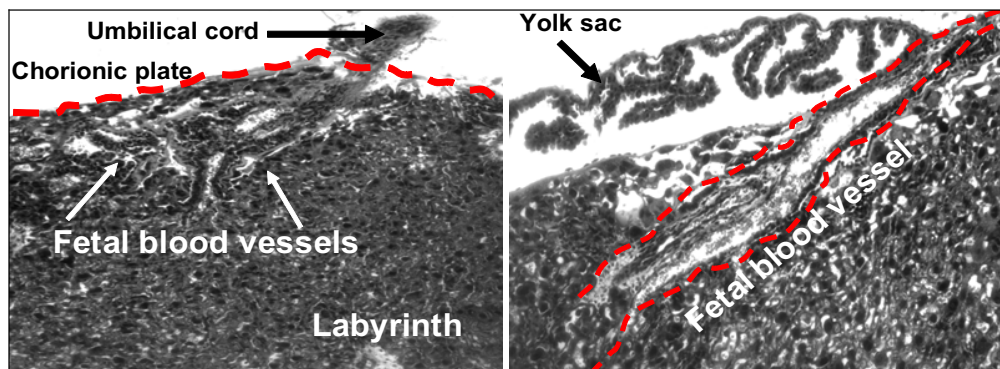
giant cells are morphologically similar and express genes in common. Giant cells are easily recognizable in placental sections, due to their extremely large nuclei. They secrete immunosuppressive agents during implantation and also, produce angiogenic factors such as, vascular endothelial growth factor (VEGF) and proliferin that are thought to target maternal vasculature and promote development and growth of vessels to the implantation site (Adamson, 2002).

The spongiotrophoblast layer was once thought to simply be a structural zone to support the underlying villi (vascular processes), but they produce a significant amount of polypeptide hormones (Linzer, 1999; Soares, 1996). The layer is a compact cellular zone that is perfused only by maternal blood and together with the giant cells, resist to maternal decidua aggressively (Zuckermann, 1986). Glycogen trophoblast cells appear within the spongiotrophoblast layer starting after about E12.5. Later, they invade into the uterus in a diffuse interstitial pattern and continue to express markers typical of spongiotrophoblast but not of trophoblasts giant cells. The developmental origin of these cells is not clear, though it is likely that they represent a specialized subtype of spongiotrophoblast cell (Adamson, 2002).

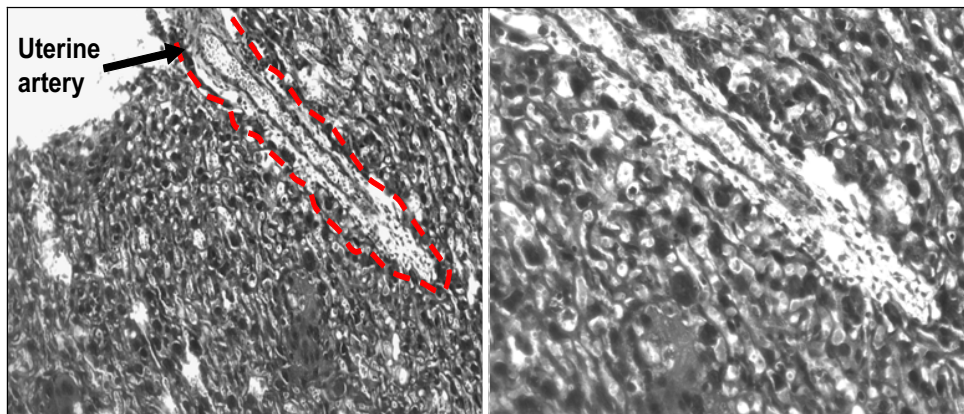


**Fig.1.4.2b Mouse placental layers illustration – a cross-section of mouse healthy term placenta.** Developed placenta contains four main layers: (i) the *giant cell layer*, (ii) the *spongiotrophoblast layer* so-called *junctional zone* and few *glycogen trophoblasts cells* (forming together the *feto-maternal interface*), (iii) the *labyrinth layer* (*placental barrier*), and (iv) the *chorionic plate*, the closest placenta area to the fetus (contains *trophoblast stem cell layer* until pregnancy term). Magnification of 10x (Patrascan, C.C. - current doctoral study)

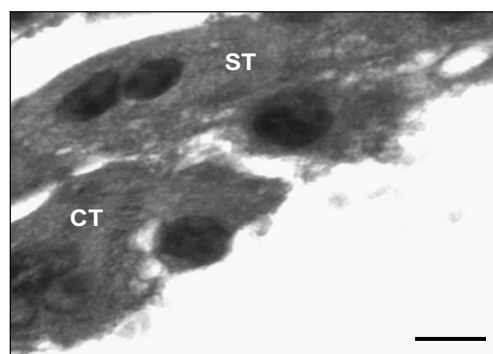




**Fig.1.4.2c Mouse chorionic plate illustration** – under chorioamnion, trophoblast stem cell layer is relived together with fetal blood vessel derived from umbilical cord (Patrascan, C.C. - current doctoral study). Magnification of 10x.



**Fig.1.4.2d Central uterine artery lined by trophoblasts cells invading decidua, placental bed in opposed direction to fetal blood vessel** – illustration with two different magnifications of 10x and 20x by light microscopy (Patrascan, C.C. - current doctoral study)



**Fig.1.4.2e Mouse placenta trophoblasts layers.** They are attached one to another forming together with fetal endothelium the placenta barrier. Illustration with high magnification of 60x by light microscopy; **CT** - Cytotrophoblast **ST** – Syncytiotrophoblast (Patrascan C.C. - current doctoral study)



Syncytiotrophoblast cells form the nutrient transport surface within the labyrinth layer of the rodent placenta. They arise from the fusion of trophoblast cells that have left the cell cycle (Cross, 2000), therefore the syncytiotrophoblast cells contain multiple diploid nuclei. Differentiation of these cells does not begin until after attachment of the allantois to the chorion at E8.5 implying that signals from the allantois may provide a signal to initiate the process. The differentiation is initiated and controlled by the activity of the glial cells missing homolog 1 - Gcm1 transcription factor (a chorion-specific transcription factor). Gcm1 mRNA expression occurs in small clusters of cells within the chorion layer as early as E7.5, a layer that is comprised of trophoblast stem cells. The sites of expression later coincide with sites in the chorion where differentiation and villous morphogenesis begin (Anson-Cartwright, 2000; Basyuk, 1999). At cellular level, Gcm1 is sufficient to promote differentiation of trophoblast stem cells towards the syncytiotrophoblast and to block their ability to differentiate into trophoblasts giant cells (Hughes, 2004).

Trophoblast stem cells emerge from the polar trophectoderm that overlies the inner cell mass in the blastocyst. They proliferate in response to close contact with the inner cell mass, now known to be mediated by fibroblast growth factor-4 (FGF4) (Rossant, 2001). FGF4 continue to be expressed by the embryonic ectoderm after implantation and indeed FGF4 - dependent trophoblast stem cells lines can be isolated from the adjacent trophoblast layer, so-called chorion trophoblast or extraembryonic ectoderm. Trophoblast stem cells can proliferate in culture for many generations and differentiate into differentiated trophoblasts cell subtypes, both in vivo and in vitro indicating that they are multipotent. Trophoblast stem cells lines resembling those derived from blastocysts or early post-implantation chorion have not been successfully isolated from tissues older than E7.5-8.0 (Takana, 1998; Uy, 2002; Hemberger, 2004). This suggests that growth of the placenta after E7.5 must be due to proliferation and differentiation of a distinct trophoblasts progenitor cell type. Thus, trophoblast stem cells are induced to proliferate and maintain their stem cell phenotype through the Fibroblast growth factor receptor 2 (Fgfr2), (Takana, 1998). The identification of

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FGF4 as a critical factor was based on the findings that mutation in both the Fgf4 gene, which is expressed by embryonic ectoderm (Feldman, 1995; Goldin, 2003) or in the FGF receptor gene 2 such as Fgfr2, which is expressed in trophoblast (Arman, 1998), results in failure of trophoblast proliferation. The TGF- $\beta$  related growth factor Nodal is required to maintain trophoblasts stem cell (TSCs) fate by cooperating with FGF signaling (Guzman-Ayala, 2004).

### 1.4.3 Placental functions

Is remarkable how the placenta a single organ, acts functionally as several organs: kidney, lungs, liver and gut, with the main functions resembling transport, respiratory, metabolic, endocrine and immunological. The placenta supplies nutrients to fetus and delivers waste from him, being also responsible for water and oxygen exchange. Placenta cells produce several steroids and peptide hormones, growth factors, cytokines, and other bioactive factors, and are able to metabolize at least some of these products (Forouzan, 2003). The total of all of these placental functions could be viewed as various brands of protection, against malnutrition, dehydration, suffocation, toxic metabolites and/or infections (Lacroix, 2002).

Respiratory function – in the fetus, the lungs are collapsed therefore, oxygen is provided through the placenta. It allows the passage of oxygen and nutrients from the mother's blood through placental barrier to the baby's blood. From here, it flows through the umbilical cord into the baby. The right side/left side circulation of the newborn is not the case in the fetus. With the lungs collapsed, there is no need for the right side of the heart to send blood to the lungs, because the blood is already oxygen rich, thanks to the placenta. Instead, there are two short cuts that allow the blood to bypass the fetal lungs. One is called the ductus arteriosus and the other is the foramen ovale. The ductus arteriosus *steals* blood normally routed to the lungs and lets it flow straight into the aorta and to the rest of the body. The foramen ovale is actually a hole in the middle wall of the heart itself, allowing blood in the right side to flow through the wall into the left side and out,

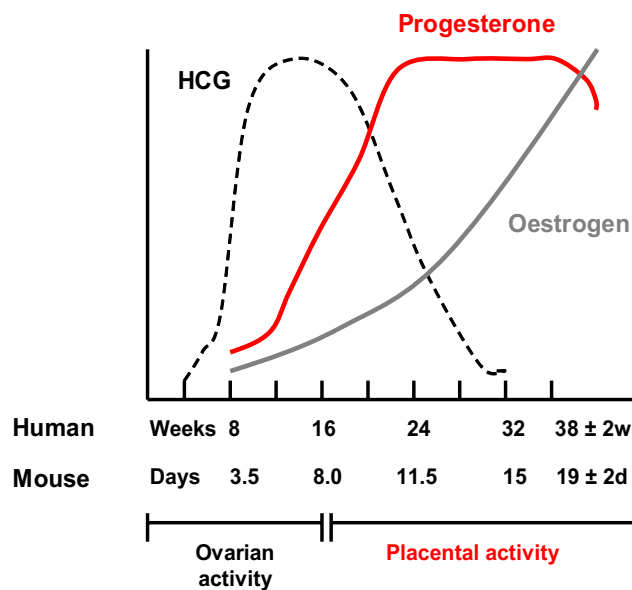
likewise, to the rest of the body. In both cases, the lungs are bypassed (Gerard, 1999). The fall in pressure as the maternal blood enters the placenta and the resulting slow flow supports the feto-maternal interchange. Maternal blood has a relatively high O<sub>2</sub> and a low CO<sub>2</sub> content. The passage of O<sub>2</sub> to the fetus and of CO<sub>2</sub> to the mother occurs by a process of simple diffusion. In addition, fetal haemoglobin has a greater ability to take up O<sub>2</sub> than adult haemoglobin does.

Transport and metabolic functions – placenta synthesizes glycogen, cholesterol and fatty acids, which serve as sources of nutrients and energy for the embryo and fetus. Carbohydrates, simple lipids and proteins are all transferred across the placenta. This occurs either by a diffusion process for glucose, but less permeable to fructose and other common disaccharides, or by special transport mechanisms – pinocytosis for amino acids (transporter proteins and peptides). The transfer of maternal antibodies (mainly IgG) is important in providing passive immunity to the newborn. Another maternal protein – transferrin – carries the iron ions (Fe<sup>3+</sup>) to the placental surface from there it is actively transported into the fetal tissues.

Excretory function – Urea is present in the same concentration in both fetal and maternal blood - the fetus eliminates carbon dioxide (CO<sub>2</sub>) and waste materials (e.g. urea and bilirubin) into the maternal circulation.

Endocrine function - The placenta produces: (i) human Placental Growth Hormone (hPGH), (ii) Insulin-like Growth Factor (IGF), (iii) Relaxin, (iv) human Chorionic Gonadotrophin Hormone (hCGH), (v) human Placental Lactogen Hormone (hPLH), (vi) Endothelial Growth Factor (EGF), (vii) Estrogens and Progesterone (Fig.1.4.3).

Steroids hormones easily cross the placental barrier, but protein hormones are much more poorly transported (but maternal thyroid hormone gains slow access to the fetus and fetal insulin can reduce symptoms of maternal diabetes).



**Fig.1.4.3 Placenta endocrine functions** (according to: Miller A., Clander R., Obstetrics illustrated, Handbook 4<sup>th</sup> Edition, 1989 and Kaufman MH. And Bard, JBL., 1999. From fertilization to implantation. The anatomical basis of mouse development, Academic Press, Harcourt Brace & Co., Publishers and Patrascan C.C. current doctoral study)

#### 1.4.4 Amniotic fluid: source and composition

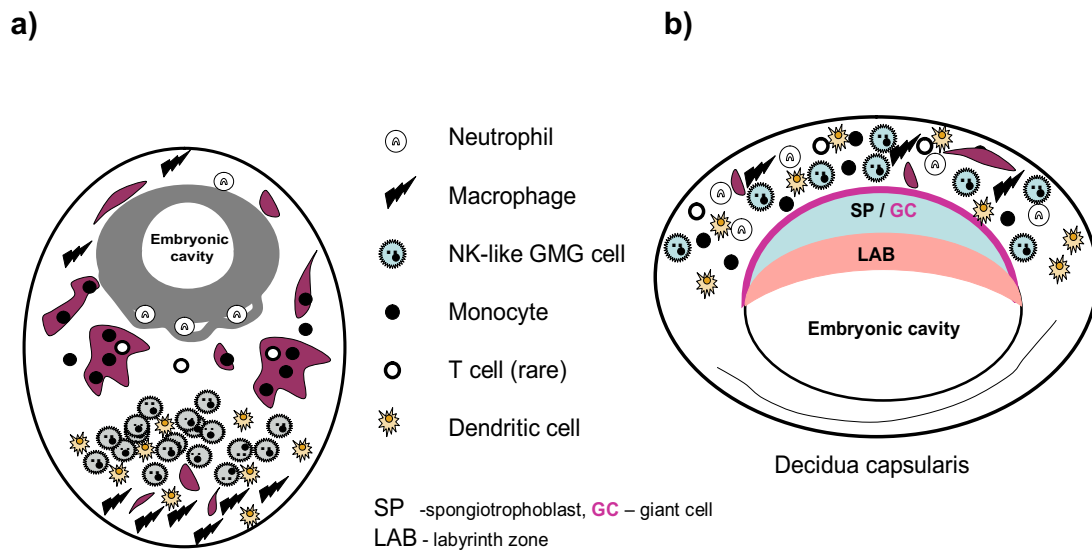
Amniotic fluid composition is not a simple *transudate*. The substances concentration of amniotic fluid are the same as those found in maternal plasma, although some may rise or fall in concert with the maternal blood. As pregnancy progresses the fetal urine is relatively greater component of the amniotic fluid. Therefore, the content of it may reflect fetal renal function. The amniotic fluid volume increases up to the 37<sup>th</sup> week (human) and 16<sup>th</sup> day (mice) falls slightly up to term and then more rapidly thereafter. The fluid is replaced every 3 hours but it may derive from several sources, mainly from amnion. Amniotic fluid source, results from both sides maternal - fetal circulation and amnion via three paths: (i) directly from maternal circulation; (ii) by diffusion through umbilical cord; (iii) mainly secreted by amnion membrane. Also, the creatinine level which rise sharply at about 38<sup>th</sup> week in human pregnancy, have been used as a test of fetal maturity. Furthermore, in the composition of amniotic fluid the chorionic gonadotrophin, placental lactogen, estrogens, progesterone and hydroxycorticosteroids are found in considerable quantities. Prostaglandins of two types are found: type E, in early pregnancy and type F, at term. Also, other

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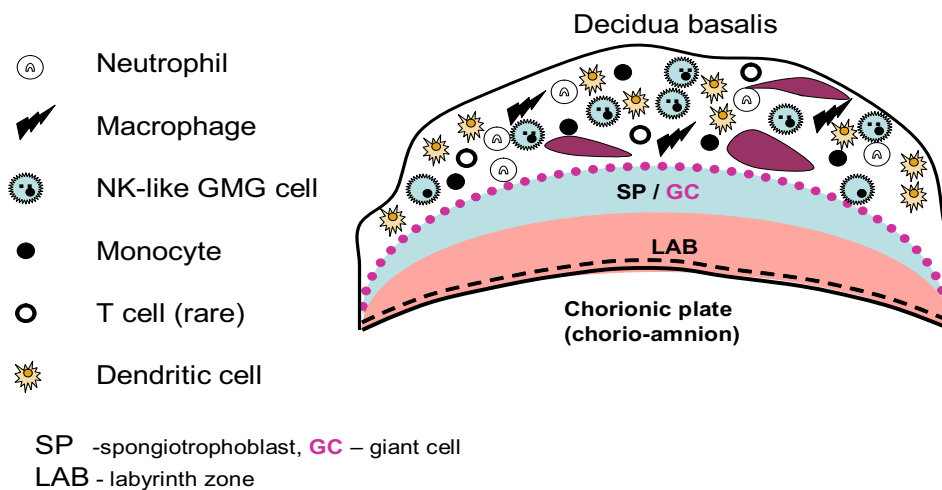
cell types are found in early pregnancy, such as: eosinophils and basophils. They are derived from fetal skin, mouth, vagina and bladder.

#### **1.4.5 Interactions between trophoblasts and other cell types in both mammalian species: human & mouse**

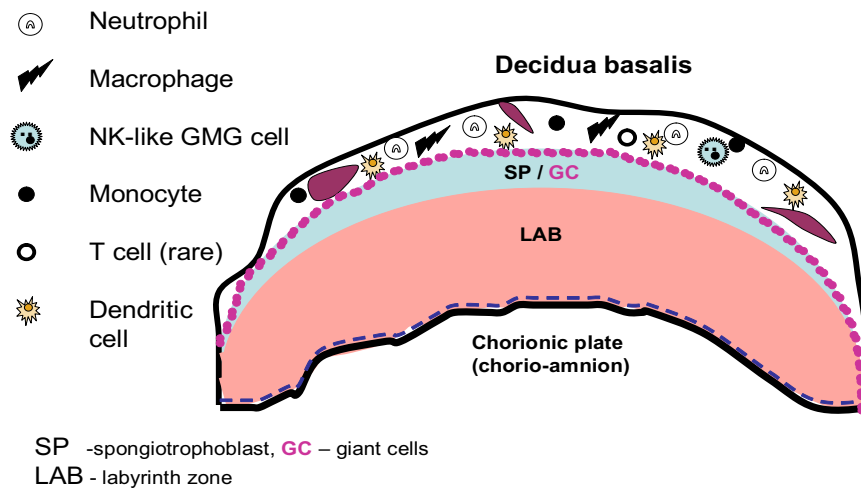
In human placental villi, proliferating trophoblasts are in close contact with the villous stroma essentially composed of fetal fibroblasts. Fetal fibroblasts promote the migration of trophoblasts through the secretion of insulin-like growth factor-1 (IGF-1), which promotes the migration of IGF-1 receptor positive CTB in trophoblast columns (Aplin, 2000; Lacey, 2002). In the decidua, the extravillous invasive trophoblasts (EVT) are in close contact with decidual cells as well as with maternal leukocytes such as uterine natural killer cells (uNKs), APCs, macrophages and T cells. These maternal cells secrete a large range of factors (including ECM, growth factors, cytokines, proteases and inhibitors) that modulate trophoblast invasion. But, the precise mechanism by which the maternal immune system deals with fetal derived antigens during pregnancy is still not completely determined. The mostly requirement for maternal APC to present antigens to fetal lymphocytes makes these cells an integral part for the induction of immunological responses. Therefore, it could be assumed that APC at the foeto-maternal interface would be ideally located for presenting fetal antigens in an inducing tolerance pathway. Endometrial tissue and decidualized endometrium in humans and rodents contain numerous leukocytes with the morphology and the phenotype of APC. It is clear that at least three populations of APC, the macrophages, dendritic cells and mature monocytes-derived APC could be found in the decidua of pregnant uterus (Fig. 1.4.5a, b, c, d).



**Fig. 1.4.5a, b Maternal cells of pregnant decidua. Mouse placenta 1<sup>st</sup> trimester of pregnancy, respective E 7.5-9.5 (embryonic day)** (according to: Kruse A. et al., 2005. Regulation of leukocytes recruitment to the murine maternal/fetal interface. Immunology of pregnancy. Chem. Immunol Allergy, Basel, Karger, vol 89, pp 105-117)



**Fig. 1.4.5c Maternal cells of pregnant decidua. Mouse placenta E 10-13.5 (embryonic day).** (according to: Kruse A. et al., 2005. Regulation of leukocytes recruitment to the murine maternal/fetal interface. Immunology of pregnancy. Chem. Immunol Allergy, Basel, Karger, vol 89, pp 105-117)



**Fig. 1.4.5d Maternal cells of pregnant decidua. Mouse placenta E 17.5-20 (embryonic day)**  
(according to: Kruse A. et al., 2005. Regulation of leukocytes recruitment to the murine maternal/fetal interface. Immunology of pregnancy. Chem. Immunol Allergy, Basel, Karger, vol 89, pp 105-117)

The specialized APC present fetal antigens derived from the invasive trophoblasts to the maternal immune system. It seems possible that intradecidual microenvironment and cellular interactions decide whether APC will acquire characteristics and functions of classical antigen-presenting mature dendritic cells (DC) with T activating features, or arrest the APC in an immature state which is thought to mediate tolerance induction (Lutz, 2002). It is remarkable that many of the factors described so far to promote tolerogenic DC, are present in abundance in the decidua. Hopefully, further research on decidual APC will shed light on how these cells help to create the delicate balance between tolerance to fetal antigens and immunity to hostile agents (Kämmerer and Markert, 2005).

#### 1.4.6 Maternal immune response throughout pregnancy and immunosuppressive agents of the placenta

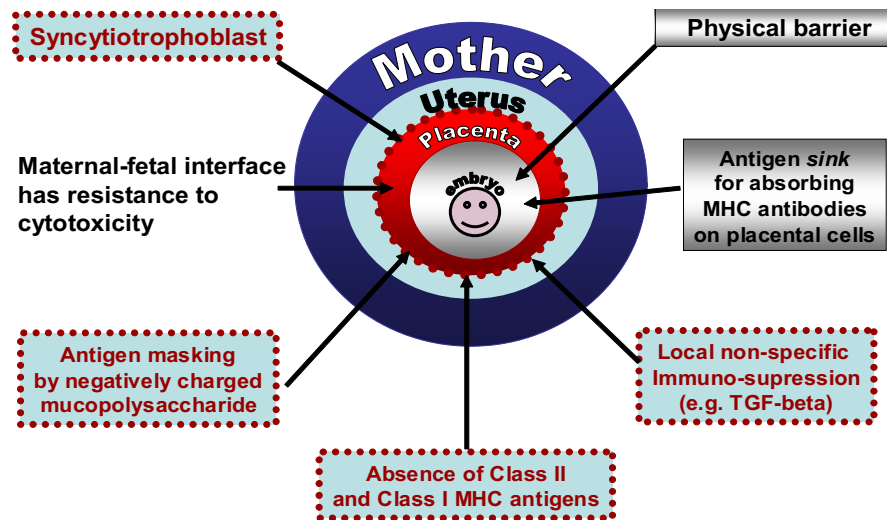
The close association of fetal and maternal tissues involves the maternal immune system in direct contact with foreign fetal antigens. Because the placenta is not considered a *classical privileged site*, mother becomes sensitized to paternal

antigens during pregnancy (van Kempen, 2001) as evidence by alloantigen-specific alterations in maternal T and B cell phenotypes (Jiang, 1998; Tafuri, 1995). Numerous studies have demonstrated that upon recognition of fetal antigen, immunoreactive T and B cells are deleted or down-modulated throughout pregnancy (Ait-Azzouzene, 1998; Zhou, 1998). Regulation of the maternal innate immune system in response to pregnancy is also critical for fetal survival (Hunt, 2000; Sacks, 1999). Indeed, it appears that, instead the maternal immune system is involved in intimate interaction with the fetal-placental entity. Often it has been hypothesized that the nature and constituents of the maternal immune response during pregnancy is modulated by the fetus (Dang, 2001). In human - fetal extravillous cytotrophoblasts (in mice – trophoblast giant cells) at the maternal-fetal interface lack classical MHC class molecules (Faulk, 1976; Rodriguez, 1997). Instead these trophoblasts in human express a MHC class Ib molecule, so-called human leukocyte antigen G (HLA-G), which is thought to provide fetal protection from maternal uNKs – mediated lysis (Kovats, 1990; Lanier, 1999). In mice, the cloning of a new nonclassical MHC class Ib gene, termed blastocyst MHC, has been described that is expressed in both blastocyst and placenta (Sipes, 1996). This gene resembles human HLA-G in structure which, unlike classical MHC I molecules may play an important role in immune surveillance to allow the maternal immune system to recognize virally infected trophoblasts cells (Wei, 1990).

Other studies have shown the role of these molecules in protecting trophoblasts from uNK cell-mediated lysis by allowing trophoblasts to be recognized as self. Nevertheless, the close association of maternal and fetal-maternal tissues exposes the maternal immunity to paternal derived antigens and many studies are focusing on how this mechanism occurs (Erlebacher, 2001; Loke, 2000).

Placenta apparently evades the maternal immune system by expression of *immunosuppressive agents*. Specific trophoblasts cell types have been shown to produce progesterone, metalloproteases, and inhibitors of complement (Cross, 1994; Munn, 1998; van Vlasserlaer, 1984), (Fig.1.4.6).





**Fig.1.4.6 Maternal immune system influences the development of the child's adaptive immune response.** The intrauterine environment correspond to a physical and biochemical support and it is not likely that the maternal immune system accepts a pregnancy just due to either a lack of fetal antigen presentation or from maternal immunological *ignorance* of the pregnancy. Placental tissue acts as an immunologically like-tissue and monitors the development of pregnancy, leading it into a  $T_H2$  immunity direction (the prenatal conceptual immunity dominance)

The complement inhibitor - Crry, is a member of a family of ubiquitously expressed molecules that negatively regulates complement components C3 and C4 (Molina, 2002). Disruption of Crry lead to deposition of complement associated with placental inflammation, leading to embryonic lethality. In human placenta, trophoblasts expressed complement regulatory proteins, presumably to circumvent complement-mediated lysis as well (Holmes, 1992). Expression of indoleamine 2, 3-dioxygenase (IDO), a tryptophan-catabolizing enzyme is shown to be present in trophoblasts. IDO is though to reduce local concentration of tryptophan below the threshold required for normal T cell function at the fetal-maternal interface (Munn, 1998; Munn, 1993; Kamimura, 1991). In the absence of tryptophan, cell cycle progression is stopped at a mid-G1 arrest point in T cells (Munn, 1999). Other immunosuppressive agents secreted at the maternal-fetal interface such as prostaglandin  $E_2$ , TGF- $\beta$  and IL-10 have a fetal protective role against maternal lymphocytes attack (Wegmann., 1993; Clark, 1990; Clark,

1998). Also, human placental trophoblasts express L-selectin, a carbohydrate binding protein, which mediates interactions with the uterus that may be critical in establishing a successful pregnancy (Genbacev, 2003). Immune evasion by the fetus is demonstrated by expression of FasL at the fetal-maternal interface which is thought to act by preventing passage of activated FasR<sup>+</sup> immune cells. In *gld* mice, a mutant line that lacks functional FasL, extensive leukocytic infiltrates and necrosis at the fetal-maternal interface are observed from E10.5 resulting in increased resorption and smaller litter sizes (Hunt, 1997; Suda, 1995).

### 1.5 Introduction to the biology of Toll-Like Receptors (TLRs) and pathogen-associated molecular patterns (PAMPs)

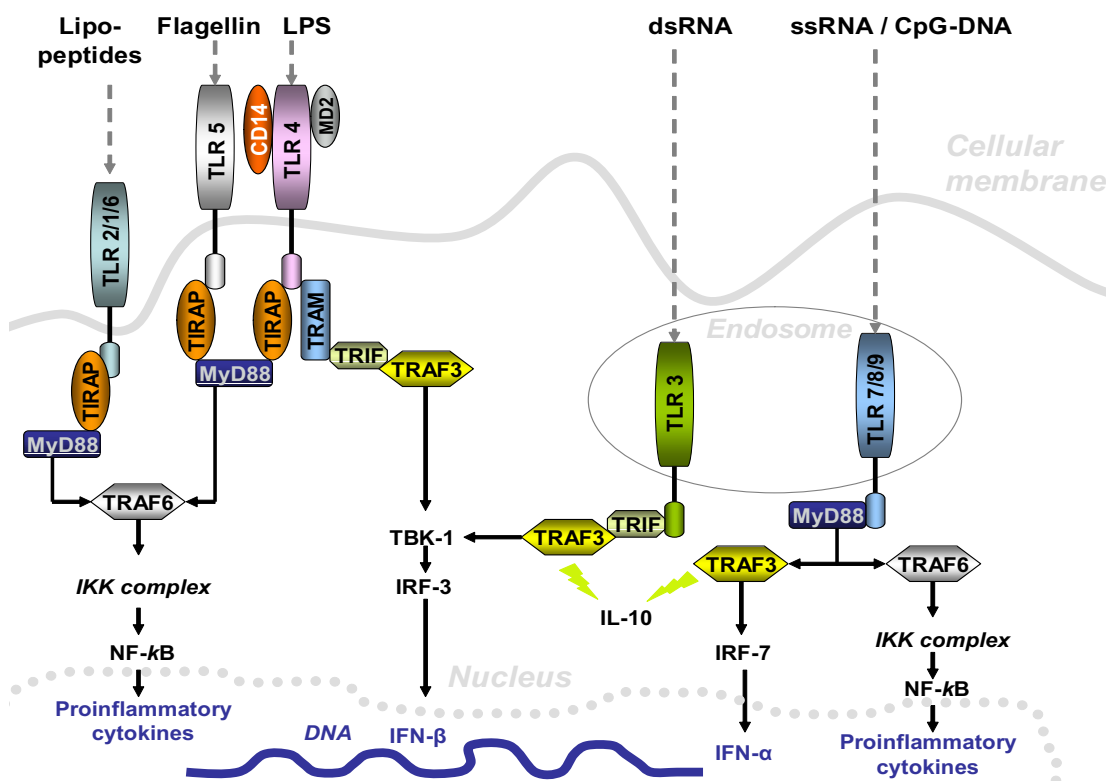
Toll-like receptors are type I transmembrane proteins that are evolutionary conserved between insects and vertebrates (Rock, 1998). In *Drosophila* fly, *Toll* was first identified as an essential molecule for dorsal-ventral patterning of the embryo and subsequently as a key molecule for the antifungal immune response in the adult animal (Anderson, 1985; Lemaitre, 1996). A homologous family of Toll receptors, termed Toll-like receptors (TLRs) exists in vertebrates (Rock, 1998). Up to now, 13 members (TLR1-13) have been reported being fundamental in recognition of pathogen associated molecular patterns (PAMPs). The family of TLR recognizes broad and diverse PAMPs from different pathogenic origins such as bacteria, viruses, fungi or protozoan parasites (Tabeta, 2004; Takeda, 2005). One subfamily of TLRs that consists of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 is expressed on the surface of cells and recognizes diverse structures. In contrast, the other subfamily is formed by TLR3, TLR7, TLR8 and TLR9 that are localized inside the cell in the endoplasmic reticulum and endosomes or lysosomes, where these receptors recognize bacterial and viral nucleic acids. Therefore, different TLRs present diversity within PAMPs recognition. Lipoteichoic acid (LTA) - an amphiphilic negatively charged glycolipid and lipoproteins – is a strong immune stimulator and activates TLR2 (Schwandner, 1999; Takeuchi, 1999). TLR2 dimerises with TLR1 and TLR6 allowing a

discrimination of differences within the lipid part of lipoproteins. The TLR2/TLR1 heterodimer recognizes triacylated lipopeptides, whereas the TLR2/TLR6 complex is activated by diacylated lipopeptides (Alexopoulou, 2002; Ozinsky, 2000; Takeuchi, 2001; Takeuchi, 2002). TLR3 recognizes double-stranded RNA (dsRNA) and the synthetic analog polyinosine-polycytidilic acid (poly I:C) and induces type I IFN (Alexopoulou, 2001). dsRNA is generated as an intermediate during the replication cycle of single-stranded RNA (ssRNA) or DNA viruses (Matsumoto, 2004). Since dsRNA is a common viral PAMP, TLR3 could be the key receptor in an antiviral immune response. Furthermore, the endotoxin lipopolysaccharide (LPS), a compound of the outer cell membrane of Gram<sup>-</sup> bacteria is a very potent PAMP among the cell wall components. The active component of the LPS a lipid fraction - Lipid A - is an immune activator molecule (Alexander, 2001). TLR4 is the key molecule of LPS induced signaling (Poltorak, 1998) and utilizes several cofactors and adaptor molecules for efficient recognition. LPS associates first with LPS binding protein (LBP) and then with soluble sCD14, a glycosyl-phosphatidyl-inositol (GPI) anchored protein (Heumann, 2003). This complex binds to the lymphocyte antigen 96 (MD2) and associates with TLR4 which leads to its aggregation and subsequent signaling (Poltorak, 1998; Shimazu, 1999). Many pathogens are motile and use a flagellum as the motility apparatus. The major component of the flagellum is flagellin, an activator of TLR5 (Hayashi, 2001). TLR5 recognizes a domain D1 of flagellin that is relatively conserved among various species (Smith, 2003). Single-stranded (ss) guanosine and/or uridine rich RNA and ssRNA viruses such as Influenza virus, Vesicular Stomatitis Virus (VSV), Newcastle disease virus (NDV) are recognized by the complex TLR7/TLR 8 (Diebold, 2004; Heil, 2004; Lund, 2004; Kato, 2005). Both genes are homologous to each other and are located on the X chromosome (Chuang, 2000; Du, 2000). TLR9 recognized bacterial genomic DNA. The stimulatory effects of bacterial DNA is due to the presence of unmethylated CpG dinucleotides in a particular base context named CpG-motif. The immunostimulatory effects of bacterial DNA can be mimicked by synthetic oligodeoxynucleotides containing a CpG-motif (CpG-ODN), (Krieg, 1995).

### 1.5.1 TLR signaling pathways: the repercussion on T<sub>H</sub>1/T<sub>H</sub>2 immunity

TLR - mediated signaling is initiated after ligand binding which presumably leads to receptor dimerization. The receptors undergo conformational change and recruit adapter molecules to their intracellular domain termed Toll/IL1-receptor like domain (TIR domain) which is shared by the TLR and IL1-receptor signaling pathway (Medzhitov, 1997; O'Neill, 1998). Four different adapter molecules have been identified: Myeloid differentiation protein 88 (MyD88), (Wesche, 1997; Adachi, 1998; Medzhitov, 1998), TIR-associated protein (TIRAP)/MyD88-adaptor like (MAL), (Fitzgerald, 2001; Horng, 2001), TIR-domain containing adaptor protein inducing IFN- $\beta$  (TRIF)/TIR-domain containing molecule 1 (TICAM 1) and TRIF-related adaptor molecule (TRAM), (Yamamoto, 2002; Oshiumi, 2003; Yamamoto, 2003a). Moreover, MyD88 and TRIF are important for the activation of distinct signaling pathway and lead to the production of proinflammatory cytokines and type I IFN. The adapter protein TIRAP/MAL is required for recruiting MyD88 to TLR2 and TLR4 along with TRAM, a TLR4 specific adaptor, which bridges TLR4 and TRIF (Kagan, 2006; O'Neill, 2006). MyD88 is the "master" adapter molecule, a protein utilized by TLRs, except TLR3. MyD88 is recruited to the cytoplasmic TIR domain through interaction with its TIR domain. Recruitment of MyD88 is followed by engagement of IL1 receptor associated kinase 4 (IRAK-4) and IL1 receptor associated kinase-1 (IRAK-1) which is phosphorylated by IRAK-4. Moreover, phosphorylated IRAK-1 associates with TNF receptor associated factor-6 (TRAF-6), whilst oligomerization leads to activate of the transcription factor NF- $\kappa$ B (Cao, 1996; Muzio, 1998; Li Q, 2002). TLR3 and TLR4 utilize TRIF to produce IFN- $\beta$  and IFN inducible genes (Hoebe, 2003; Yamamoto, 2003b). TRIF interacts with receptor-interacting protein-1 (RIP-1) and TANK binding kinase 1 (TBK-1) to initiate NF- $\kappa$ B activation and IRF-3, IRF-7 phosphorylation (Fitzgerald, 2003; Sharma, 2003; Meylan, 2004). Phosphorylated IRF-3 and IRF-7 form homodimers, translocate into the nucleus and regulate the expression of IFN-inducible genes by binding to interferon stimulated response element (ISRE), (Sakaguchi, 2003; Honda, 2005). In pDC,

IFN $\alpha$  production via TLR7 and TLR9 is strictly dependent on MyD88 and contrasts the TRIF dependency of IFN production via TLR3 and TLR4. In general, TLR-mediated activation of immune cells leads to the production of IFN $\alpha$ , proinflammatory cytokines such as TNF $\alpha$ , IL-1 and IL-6 as well as the regulatory cytokines IL-12 and IL-18 promoting T<sub>H</sub>1 differentiation (Wagner, 2001), (Fig1.5.1).



**Fig.1.5.1 TLR signaling pathways.** Activation of TLR induces secretion of proinflammatory cytokines, type I IFN and anti-inflammatory IL-10 depending on a specific TLR, cell type and the adaptors used for signal transduction MyD88, TIRAP, TRAM, and TRIF. The relationship of TLRs by their ligands leads to the activation of a signaling cascade with subsequent induction of genes that are involved in the immune response against pathogens. In general, three major pathways are activated: (i) the activation of the transcription factor NF- $\kappa$ B, which acts as a *master switch* for inflammation, (ii) activation of the MAP kinases p38 and Jun amino-terminal kinase (JNK), which participate in increased transcription and (iii) the type I IFN production via IFN regulatory factors (IRFs) (according to: Bauer, S. et al., 2007. Immunobiology of toll-like receptors in allergic disease. Immunobiology 212, pp 521-533)

Accordingly, MyD88<sup>-/-</sup> mice lack of TLR mediated signaling resulted in increasing T<sub>H</sub>2 response with strong IgE production (Schnare, 2001). The importance of TLR signaling in T<sub>H</sub>1 immune responses implies that variations in TLR expression and activity may influence the development and severity of allergic diseases. Also, for some TLR, such as TLR2 and TLR5 conflicting results have been observed regarding the induction of a T<sub>H</sub>1 or T<sub>H</sub>2 polarization depends on the purity of the ligand (Agrawal, 2003; Didierlaurent, 2004; Velasco, 2005). Thus, the concentration of LPS may settle on what type of immune response is initiated. Low concentrations may induce MyD88 independent T<sub>H</sub>2 activation, whereas higher LPS concentrations induce a MyD88 dependent T<sub>H</sub>1 response (Eisenbarth, 2002; Kaisho, 2002). For the MyD88-independent immune activation pathway via TRIF and TLR3, a strong induction of a T<sub>H</sub>1 response has been reported (Wang, 2002).

### **1.5.2 TLR signaling in trophoblasts cells throughout normal and pathological pregnancies**

Toll-like receptors (TLR or TLRs) are widely expressed throughout the immune system, specifically in the innate immune system. These proteins can also be expressed by non-immune cells, particularly if such cells are able to contribute to an inflammatory response, and most tissues express at least one TLR (Zarembek, 2002). Toll-like receptor expression by mucosal systems is important for host defense against pathogens (Gewirtz, 2003; Yuan, 2004). While most studies have focused on the intestinal and respiratory tracts, there is growing evidence that the mucosal epithelium of the female reproductive tract (FRT) is also an important immunological site (Wira, 2004; Quayle, 2002). Hence, Toll-like receptors are expressed by endometrial epithelium and the epithelial cells (e.g. placenta) of the lower reproductive tract and furthermore, these cells are able to respond to microorganism through these receptors (Fichorova, 2002; Schaefer, 2004; Young, 2004). During pregnancy there is a strong immunological presence at the fetal-maternal interface on placenta tissue, particularly by the innate

immune system (Mor, 2002). The role of the immune system at the fetal-maternal interface is thought to facilitate implantation and placental development, whilst promoting fetal tolerance and protection (Audus, 2002; Moffett-King, 2002). However, a certain level of host defense at this site is also required. While immune cells such as macrophages and NK cells are present at the maternal-fetal interface (Mor, 2002), they may not be the only cells able to respond to bacteria. In addition to the classical immune cells, placental cells – trophoblasts – may also have the potential to function as a component of the innate immune system (Guleria, 2000). Currently, little is known about the role of Toll-like receptors during pregnancy. Normal term placental tissue has been shown to express TLR1 to TLR10 in human and TLR1 to TLR13 (except TLR10) in mouse at the mRNA level. Syncytiotrophoblast and intermediate trophoblast of term placenta express at the protein level, TLR2 and TLR4 (Holmlund, 2002). In contrast (Kumazaki, 2004) shown in human, that the TLR4 positive placental cells might be term extravillous and intermediate trophoblasts. Such contradictory findings might be a result of the type of antibody that detects TLR4 complexes with the adaptor protein MD-2 whilst others used an antibody that detects TLR4 alone (Holmlund, 2002). Nonetheless, these findings do suggest that trophoblasts cells may interact with microorganisms present already at the implantation site being able to initiate an immune response. Therefore, the trophoblasts function as an active member of the innate immune system (Guleria, 2000). Abrahams et al. demonstrated the expression of TLR2 and TLR4 mRNA in first trimester placental tissue. They could show that the villous cytotrophoblast and extravillous trophoblasts cells from first trimester trophoblast cell populations are expressing these receptors. Instead, first trimester syncytiotrophoblasts do not express these TLRs suggesting that the placenta serves as a highly specialized barrier, protecting the developing fetus against infections (Abrahams, 2004). These studies of the lack of TLR expression by the outer trophoblasts layer – syncytiotrophoblast - is analogous to studies of mucosal epithelial cells of the intestinal tract which have been shown to express TLR4 and TLR5 only on their basolateral side (Gewirtz, 2001). These cells will only respond to a

bacterium that has invaded the basolateral compartment from the apical side. Since a pathogen is characterized as a microorganism that breaches certain physical barriers, these observations have helped to explain how an immune response can be mounted against pathogenic, but not commensal bacteria. Similarly, a microorganism will only be a threat to the fetus if the TLR-negative syncytiotrophoblast cell layer is breached and the pathogen has entered either the decidual or the placental villous compartments. Therefore, the placenta can distinguish between pathogenic and commensal microorganisms during pregnancy. Thus, once a microorganism has gained access to the TLR-positive trophoblasts cells, a response may be rising but the type of pathogen and the specific receptor activated may have a significant impact on the type of response generated by the cells of the placenta. The function of Toll-like receptors at the feto-maternal interface is an area of research still at the beginning. It is known that trophoblasts cells from term placental explants can produce IL-6 and IL-8 following ligation of TLR2 or TLR4 by Zymosan or LPS (Holmlund, 2002). Treatment of term trophoblasts cells with LPS has also been shown to induce the production of nitric oxide - which has potent anti-microbial properties and MMP-2 (Nakatsuka, 2000). Studies on first trimester trophoblasts have shown that treatment with LPS induces the production of G-CSF and RANTES (Svinarich, 1996). Recently, in some studies was found that activation of TLR4 by LPS generated a classical TLR response, characterized by the enhanced production of both pro- and anti-inflammatory cytokines (Abrahams, 2004). Together, all these studies suggest that trophoblasts cells can indeed function similarly to cells of the innate immune system, by recognizing and responding to components of microorganisms.

Since clinical studies have shown an association between intrauterine infections and preterm labor, Preeclampsia and Intrauterine Growth Restriction (IUGR) (Concalves, 2002; Romero, 2003; von Dadelszen, 2003; Hsu, 1995; Arechavaleta-Velasco, 2002) some studies shown that TLR expressed at the fetal-maternal interface may play an important role in the mechanism of pathogenesis. Certain intrauterine infections during pregnancy may have either a



direct or an indirect effect upon trophoblasts cell survival, depending upon which TLR could be activated. The Gram<sup>+</sup> bacteria expressing peptidoglycan (PG) or lipoteichoic acid (LTA) may directly promote trophoblasts cell death through TLR2 signaling pathway. Recently, soluble TLR2 (sTLR2) has been identified (LeBouder, 2003). This protein may function by modulating specific TLR-mediated responses. Alternatively, soluble forms of TLR may bind to microorganisms and flag them for destruction by the complement system or by phagocytosis (Mezhdhitov, 2002). Consequently, the soluble TLR (sTLR) may provide new markers of pregnancy complications as well as a potential target for therapeutic interventions. Therefore, animal models used for pregnancy complications have been generated by the administrations of high amounts LPS (Kaga, 1996; Vizi, 2001; Benett, 2000; Baines, 1996; Orgando, 2003). LPS, through TLR4, triggers first trimester trophoblasts cells to produce high levels of T<sub>H</sub>1 cytokines, suggesting that both TNF $\alpha$  and IFN $\gamma$ , may induce trophoblasts apoptosis and fetal loss at this pregnancy stage (Yui, 1994; Garcia-Lloret, 1996; Ho, 1999; Crocker, 2001; Aschkenazi, 2002). Hence, while LPS does not directly induce trophoblasts cell death, the intense inflammatory response generated by either trophoblasts or decidual immune cells following its activation may provide an alternative mechanism for the induction of trophoblasts cell death other than pregnancy at term.

Allergic disorders including allergic rhinitis, atopic dermatitis, food allergies and allergic bronchial asthma are inflammatory diseases which employ a vary risk of factors. Their identification such as: smoking, indoor allergen exposure, RSV infection, the appearance of allergic disease lead to the identification of protective factors. Thus, based on epidemiological data *the Hygiene hypothesis* (proposed for the first time by Strachan, in 1989) shows that certain life circumstances are strongly related with protection against allergies becoming asthma associated. Remarkable evidence is that the cross-sectional study on the prevalence of allergy to schoolchildren in farmer versus non-farmer environment. Children from farmer locations have a 50% less risk of an allergy and asthma disease as children from non-farming life circumstances. An immune protection is indispensable to the higher exposure to bacterial antigens such as farming environment, unpasteurized cow's milk. These observations suggest that exposure to farming environment may influence the developing immune system leading to a reduced risk for the development of allergies. Therefore the applicable *window of opportunity* is defined by exposure to the protective environmental factors early in life, starting already in utero.

The theme of the presented doctorate's thesis is defined as: *The role of Toll-like receptors in the development of immunological tolerance in neonates*, part of the research project SFB - TR22/A18 so-called: *Mechanisms of pre- and postnatal instruction of allergic respiratory diseases in the mouse model of experimental asthma*. It has in focus the epidemiological data regarding the differences into development of immune systems from children growing-up into farming and non-farming environments.

**But how could we manage nowadays the allergies in our children?**

**What is the starting point of avoidance of these aggressive phenomena?**

Regarding these questions we hypothesized that: *The activation of Toll-like receptors in the placenta plays an important role in prenatal transmission of anti-allergic protective immunological effects from mother to fetus.*

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This hypothesis is strongly supported by other two observations that assumed:

- (i) in mother, the prenatal induction and instruction of well defined immune and placental positive cells is mediated by innate regulatory pathways (Toll-like receptors)
- (ii) the perinatal exposure to bacterial constituents induce in newborn and infant a switch of  $T_H2$ -phenotype into an anti-allergic  $T_H1$  immune response

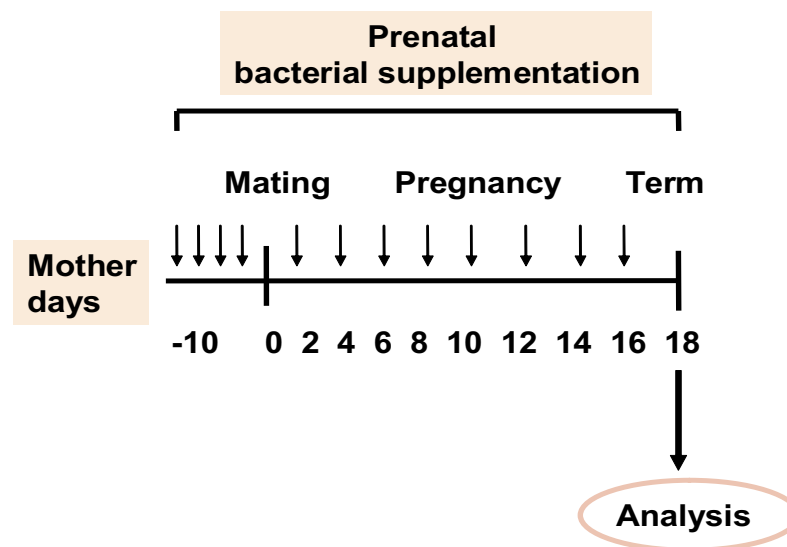
Accordingly, the aims of the study are: - to monitor the complexity of pregnancy by mimicking the farming environment in our laboratory with an established animal model, in order to compare the human and mouse morpho-pathology and placental immunology (from trophoblast stem cells – TSCs - to entire placental tissue). Regarding, pregnant mice are supplemented with Gram<sup>+</sup> bacteria (Lactobacillus rhamnosus GG or LGG), Gram<sup>-</sup> bacteria (Acinetobacter lwoffii F78 or Ac. lwoffii F78) and Gram<sup>-</sup> bacterial active component (Lipid A or LA, derived from LPS/E.coli). The ending point of the doctoral experimental work was to *design* a potential maternal anti-allergic protective molecular mechanism to the fetus. This is supported by the beneficial effects transmitted from mother to her concepti, and *converted-like* the development of  $T_H1$  immunity in the offspring.

Starting from the new insights that TLR signaling pathways expressed in placenta are responsible from the defensive/protective mechanisms against allergy disorders, we carried out perinatal (pre- and postnatal) experiments on the establish mouse model regarding to human disorders with a great technical performance of a broad of biochemical, immunological and molecular biology methods. Finally, the perinatal results add evidences that the farming environment circumstances are crucial in a well developing pregnancy phenotype and consequentially, educating the children's immunity fighting against allergies via TLR signaling pathways.

### 3.1 Design of experimental protocols for perinatal study

#### 3.1.1 Intrauterine experimental animal model – prenatal exposure to farm environment containing non-pathogenic bacteria

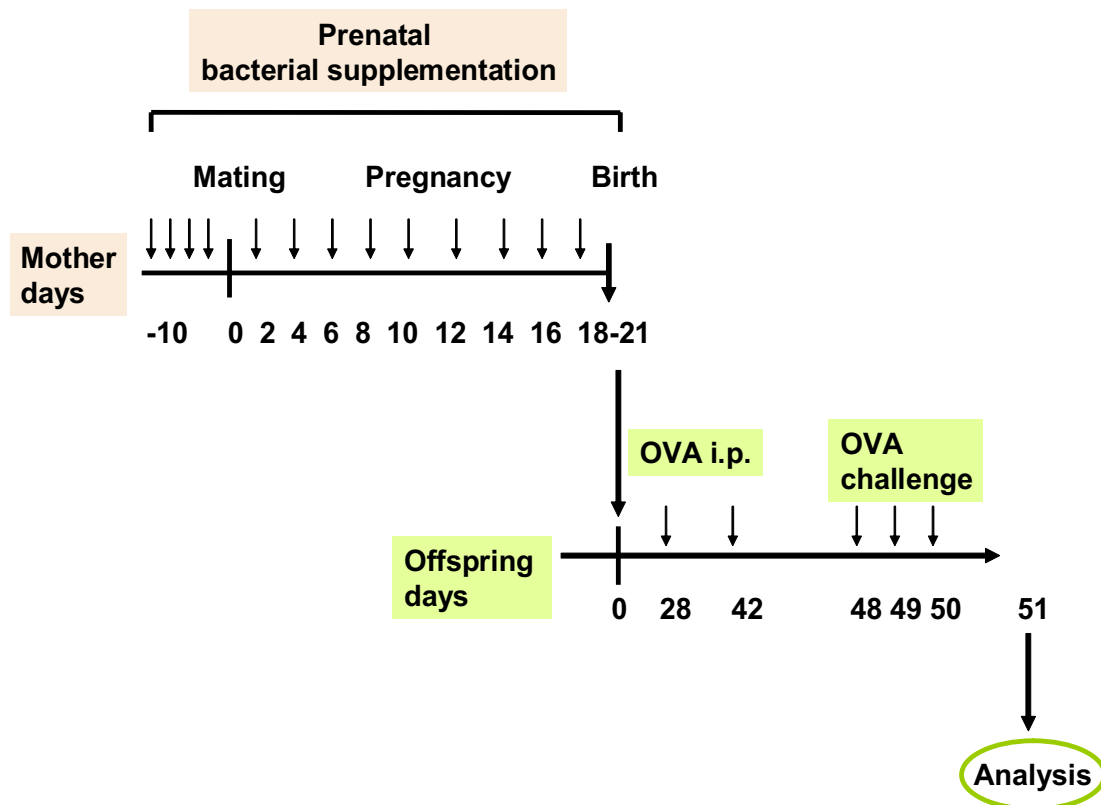
Female Balb/c strain mice, 6-8 weeks old, received either intragastric (i.g.) *Lactobacillus rhamnosus* GG - a Gram<sup>+</sup> bacteria (concentration of  $10^8$  CFU/200  $\mu$ l per application, reconstituted in PBS) and intranasal (i.n.) *Acinetobacter lwoffii* - a Gram<sup>-</sup> bacteria (concentration of  $10^8$  CFU/50 $\mu$ l per application, reconstituted in PBS) and Lipid A - the active component of LPS derived from *E.coli*, a Gram<sup>-</sup> bacteria (10  $\mu$ g/50  $\mu$ l per application, reconstituted in PBS). Maternal supplementation started 10 days before conception every second day and continued until term of pregnancy. Age-matched control animals received PBS. All females were sacrificed at term of pregnancy to perform the blood, amniotic fluid, small intestine spleen, and placenta analyses for TLR, cytokine and chemokine, and steroid hormones expression using immunological, molecular biological and biotechnological techniques (Fig.3.1.1).



**Fig.3.1.1 Experimental protocol of prenatal supplementation with non-pathogenic bacteria**  
 - *Lactobacillus rhamnosus* GG – Gram<sup>+</sup> bacteria strain, *Acinetobacter lwoffii* F78 – Gram<sup>-</sup> bacteria strain, Lipid A - the active component of LPS (a Gram<sup>-</sup> bacterial membrane component - *Escherichia coli*), whilst the age-matched control animals received PBS

### 3.1.2 Acute asthma animal model – OVA sensitisation and challenge of the offspring

Wild type female used as control and  $MyD88^{-/-}$ ,  $TLR2^{-/-}/3^{-/-}/4^{-/-}/7^{-/-}/9^{-/-}$  and  $TNFP55^{-/-}$  deficient mice female mice C57BL/6J strain, 6-8 weeks old received intranasal (i.n.) lyophilized *Acinetobacter lwoffii* F78 (concentration of  $10^8$  CFU/50  $\mu$ l per application, reconstituted in PBS). Maternal supplementation started 10 days before conception every second day being maintained until term of pregnancy. Age-matched control animals received PBS. Later after birth, asthma was induced in the young animals.



**Fig.3.1.2 Experimental protocol of acute asthma animal model – OVA sensitisation and challenge of the offspring** - *Acinetobacter lwoffii* F78 prenatal supplementation is followed by induction of acute in offspring by OVA sensitization and challenge, whilst the age-matched control animals received only PBS.

Offspring mice were sensitized twice with intraperitoneal (i.p.) injections of 200 µl Ovalbumin (OVA)/Al(OH)<sub>3</sub> per application (10 µg OVA emulsified in 1.5 mg Al(OH)<sub>3</sub> as adjuvant reconstituted in PBS) at the age of 28 and 42 days. At days 48, 49, 50 the subgroups of offspring were placed one after other into a Plexiglas chamber and exposed (challenged) to the aerosolized OVA (1% wt/vol reconstituted in PBS) for 20 minutes time exposure. Lung function analysis was carried out with respect to monitor the airway-hyper-reactivity (AHR) to β-Acetyl-Methacholine (MCh) using the non-invasive method so-called Head-out Body-Plethysmography. The middle-expiratory airflow (EF50) of bronchial responsiveness to β-Acetyl-Methacholine (MCh) was measured 24h (day 51) after the last OVA aerosol challenge. Subsequently, after 48h (day 52) the animals are euthanized by neck dislocation, the trachea is cannulated and washed 5 x 1 ml of cold PBS to obtain the bronchoalveolar lavage (BAL). The total number of leukocytes was determined by using a CasyTT Cell Counter (Schäfer System, Reutlingen, Germany). Furthermore, to obtain the alveolar cells and cell-free lavage fluid, BALF is centrifugated at 300g for 10 minutes under temperature of 4°C. Investigating the airway inflammation was important to quantify the protein production (cytokine and chemokine measurement by ELISA and CBA methods) from cell-free lavage fluids which were preserved at -80°C and then, the cellular composition of BAL fluids (total number of leucocytes, number of eosinophilic cells - granulocytes, lymphocytes, macrophages and neutrophils). Therefore, alveolar cells were suspended in 1 ml of PBS, total cell numbers were determined before the cytospins were prepared. The cytospins were dried and stained with May-Grünwald Giemsa staining method. Macrophages, neutrophils, lymphocytes and eosinophils were simple differentiated by light microscopy.

### **3.2 Mouse strains**

For the prenatal study – intrauterine animal model - female and male Balb/c strain mice, aged 6-8 weeks were obtained from Harlan-Winkelmann GmbH, Hannover, Germany, while the postnatal study required different mouse strain

such as wild type and deficient mice, in order to demonstrate the hypothesis of the study. Regarding, MyD88<sup>-/-</sup>, TLR 2<sup>-/-</sup>/3<sup>-/-</sup>/4<sup>-/-</sup>/7<sup>-/-</sup>/9<sup>-/-</sup> and TNFα<sup>-/-</sup> deficient mice and wild types littermates, C57BL/6 background were kindly obtained from different collaborators: Prof. Dr. Stefan Bauer and PD Dr. Philipp Yu (Philipps-University of Marburg/Lahn, Institute of Immunology, Biomedical Research Center – Biomedizinische Forschung Zentrum), Prof. Dr. rer. nat. Carsten Kirschning (Technical University of München and Institute for Medical Microbiology, University of Duisburg-Essen), Prof. Dr. med. Klaus Pfeffer (Heinrich-Heine-University of Düsseldorf, Institute of Medical Microbiology). Animals were maintained on germ-free conditions and OVA-free diet receiving food and water *ad libitum* being bred and experimented at the Medical Research Center and Biomedical Research Center Laboratories, Marburg/Lahn, Germany. All experimental procedures were performed in accordance with German and international guidelines, and were approved by local authorities (Regierungspraesidium Giessen).

### 3.3 Bacterial strains

*Lactobacillus rhamnosus* GG (LGG) - a Gram<sup>+</sup> bacteria strain so-called *probiotic bacterium* that was originally considered to be a subspecies of *Lactobacillus casei* (*L. casei*), but later genetic research found it to be a species of its own. LGG inhibits the growth of most harmful bacteria in the intestine. The lyophilised LGG was purchased from Valio GmbH, Helsinki, Finland.

*Acinetobacter lwoffii* F78 (Ac. lwoffii) - a Gram<sup>-</sup> bacteria belonging to the Phylum Proteobacteria. As a non-motile bacterium, *Acinetobacter* species are oxidase-negative illustrating pairs under microscopically magnification. *Acinetobacter* species are generally considered nonpathogenic to healthy people but most infections occur in immunocompromised individuals. Many of the immune activating abilities of LPS could attributes to the Lipid A unit. It is a very potent stimulant of the immune system, activating cells (e.g. monocytes or macrophages) at picogram per milliliter quantities. When present in the body at high concentrations during a Gram<sup>-</sup> bacterial infection, it may cause shock and

death by an immune overreaction. *Acinetobacter lwoffii* F78 bacterial strain was identified and kindly provided by Prof. Dr. Otto Holst - Division of Structural Biochemistry, Research Center Borstel, Leibnitz-Center for Medicine and Biosciences, Germany.

Lipid A (LA) - the active part of LPS (*E.coli* membrane component, a Gram<sup>-</sup> bacteria) consists of two glucosamine (carbohydrates/sugars) units with attached acyl chains or fatty acids, and one phosphate group on each carbohydrate. The optimal immune activating Lipid A structure is believed to contain 6 acyl chains. Four acyl chains attached directly to the glucosamine sugars are  $\beta$ -hydroxyacyl chains usually between 10 and 16 carbons in length. Two additional acyl chains often attached to the  $\beta$ -hydroxy group. Lipid A has four - C14 hydroxyacyl chains attached to the sugars, and one C14 and one C12 attached to the  $\beta$ -hydroxy groups. The biosynthetic pathway for Lipid A in *E. coli* has been determined by the work of Christian R. H. Rätz in the past 20 years (Rätz, 2002). Lipid A with a reduced number of acyl chains can serve as an inhibitor of immune activation induced by Gram<sup>-</sup> bacteria, and synthetic versions of these inhibitors are in clinical trials for the prevention of harmful effects caused by Gram<sup>-</sup> bacterial infections. On the other hand, modified versions of Lipid A can be used as components of vaccines (adjuvants) to improve their effect. Furthermore, Lipid A and/or LPS is believed to activate cells via Toll-like receptor 4 (TLR4), MD-2 and CD14 on the cell surface. Lipid A was provided from Sigma GmbH, Deisenhof, Germany.

### **3.4 Methods - RNA and DNA Analysis**

#### **3.4.1 Polymerase Chain Reaction (PCR) of RNA isolated and cDNA synthesis from maternal small intestine, spleen, placenta and trophoblast stem cells (SM10 cell line)**

RNA was isolated from maternal organs such as spleen and placenta with the TriFast lysis buffer (Pepqlab GmbH, Erlangen, Germany) according to manufacturer's instructions. Previously, 50-100 mg of organ was perfused with



cold and sterile PBS, then frozen-up in liquid nitrogen and homogenized in TriFast lysis buffer. Afterwards the mechanical disruption tissues was done using a mortar and pestle. The suspension obtained is trasfered into a liquid-nitrogen-cooled, appropriately sized tube and allow the liquid nitrogen to evaporate without allowing the sample to thaw. The tissue suspension was maintained 5 minutes at room temperature for enzymatic digestion. The phase separation process is the next step. Volume of 200 µl of Chloroform pro 1ml TriFast lysis buffer was added and the whole mixture was centrifuged for 5 minuntes at 12,000g under 4°C. The supernatant as a transparent phase contains the total RNA from the entire tissue was transferred into new 1.5 ml Eppendorf tube. The extracted RNA was precipitated using Isopropanol (absolute solution), followed by a new centrifugation for 10 minutes at 12,000g under 4°C. Then, the RNA pellet is washed out with 1 ml Ethanol 75% (v/v) pursued by a new centrifugation of 5 minutes at 12,000g under 4°C. The RNA pellet could be preserved with Ethanol 75% (v/v), in freezer at -20°C for one month or could be solubilised by solving the pellet into 60 µl Rnase-free water and incubate for 10 - 15 minutes at 55°C. Thus, the total RNA extracted from tissue was used immediately proceeding cDNA synthesis reverse transcription or preserved as biomaterial storage in freezer at -80°C for several months. Furthermore, from maternal gut and placental trophoblast stem cells (SM10 cell line) RNA was isolated by using the Rneasy Lipid Tissue Mini Kit (50) (Qiagen GmbH, Hilden, Germany), respective the Rneasy Mini Kit (50) (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. Previously, the placental trophoblast stem cells were treated with Beta-mercaptoethanol (β-ME) reconstituted in RLT lysing buffer (guanidine thiocyanate salting solution) with dilution of 1:100, in order to disrupt the cell membrane (for a number of pelleted cells  $<5 \times 10^6$  was used 350 µl of RLT lysing buffer). The contaminating genomic DNA was removed by DNase treatment (DNA-free Set; Ambion Company, Austin, TX, USA) or DNase digestion column from RNase-free DNase Set (Qiagen GmbH, Hilden, Germany). Alternatively, residual DNA could be removed by a DNase digestion after RNA extraction by using the Rneasy Cleanup Mini Kit (50) (Qiagen GmbH,

Hilden, Germany). The reason of using different kits is given by the organ constitution itself in order to obtain a high quality of RNA extracts.

The cDNA synthesis was performed by *Omniscript Reverse Transcriptase Set* (Qiagen GmbH, Hilden, Germany). This is a standard protocol for first-strand cDNA synthesis using 50 ng to 2 µg RNA. The efficiency of the synthesis is highly dependent on the quality and quantity of the starting RNA template. In this regard, the concentration of tissues isolated of total RNA was measured by Spectrophotometer at specific parameters: OD<sub>240-260nm</sub>, DF=50, integration time 5 sec, F= 40, conc.units µg/ml. For RNA sequences quality, a normal PCR was performed (TAE running buffer, RNA - Agarose powder, Sample Loading Buffer and RNA Ladder 100bp as marker from Sigma, Germany). Template cDNA generated by this Omiscript Reverse Transcriptase method is suitable for the standard PCR, real-time RT-PCR, but also, synthesis of double-stranded cDNA for cloning, labeling for microarrays, analysis of transcription start by primer extension, serial analysis of gene expression (SAGE<sup>TM</sup>), exponential RNA amplification (NASBA<sup>®</sup>, TMA), linear RNA amplification, rapid amplification of cDNA Ends (RACE).

#### **3.4.2 Real-time RT-PCR or Quantitative Reverse Transcriptase – PCR (qRT- PCR) of mRNA TLR and mRNA T<sub>H</sub>1/T<sub>H</sub>2 cytokine**

The Omniscript Reverse Transcriptase is a recombinant heterodimeric enzyme expressed in *E.coli*. The quantity of isolated RNA corresponds to the entire amount of RNA including (ribosomal) rRNA, (messenger) mRNA, viral RNA and carrier RNA present in tissue and regardless of the primers used or cDNA analysed. It was not used a separated denaturation and annealing steps necessary for some RNAs with a high degree of secondary structure. In this case, the isolated RNA was denaturated in RNase-free water reagent by incubation at 55°C for 5 minutes and placed immediately on ice. Concentration and length of the primers (Oligo-dT and gene-specific) used were optimized individually (e.g.Oligo-dT has 18 nuclotides, therefore the final concentration

used must be in the range of 0.1-1.0  $\mu$ M) that specifically hybridize to the poly-A-tail of mRNAs. For the quantification of RNAs transcripts, real-time RT-PCR was used because is a high sensitive and reliable method.

Real-time – PCR (RT-PCR) begins with reverse transcription of RNA into cDNA, followed by PCR amplification of the cDNA. The quantity of cDNA is determined during the exponential phase of PCR by the detection of fluorescence fluorophores incorporated into the PCR product such as SYBR<sup>®</sup> Green. The level of the transcripts was calculates from the number of the PCR cycles at which the threshold is exceeded, thus called "the threshold cycle" or the crossing point. Real-time RT-PCR or Quantitative RT-PCR (qRT-PCR) was performed using the QuantiTect SYBR<sup>®</sup> Green PCR Kit (Qiagen GmbH, Hilden, Germany) and listed mouse Toll-like receptors specific primers such as TLR 1 to 9 or cytokines, chemokines or other immunomodulatory molecules:

- mouse **TLR1** primer (sense 5 –TGT CCA AGC TGA GGG TCC TG-3, antisense 5-GCT TGA GGC TGA CTG TTC GG-3)
- mouse **TLR2** primer (sense 5- GCC AAG AGG AAG CCC AAG A-3, antisense 5-AAG GGC GGG TCA GAG TTC TC-3)
- mouse **TLR3** primer (sense 5- AAC AAC GCC CAA CTG AAC CC-3, antisense 5- TGC CGA CAT CAT GA GGT TG-3)
- mouse **TLR4** (sense 5- GCA GGT GGA ATT GTA TCG CC-3, antisense 5- TTC GAG GCT TTT CCA TCC AA-3)
- mouse **TLR5** primer (sense 5- ACC AAC GTC ACC CTG TTC GG-3, antisense 5- GAC CGC ATG GCT TCC TCT TC-3)
- mouse **TLR6** primer (sense 5- CGA GCC TGA GGC ATC TAG ACC-3, antisense 5-GAG CAA CTG GGA GCA GAT CC-3)
- mouse **TLR7** (sense 5- TCC CAG AGG CCC ATG TGA TC-3, antisense 5-CCA GAT GGT TCA GCC TAC GG-3)
- mouse **TLR9** primer (sense 5- GGG CCC ATT GTG ATG AAC C-3, antisense 5- GCT GCC ACA CTT CAC ACC AT-3)

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Other primers used, such as:

- mouse **IL-4** primer (sense:5'-TCA ACC CCC AGC TAG TTG TC-3, antisense 5'-TGT TCT TCG TTG CTG TGA GG-3)
- mouse **IL-10** primer (sense 5- GCA TGG CCC AGA AAT CAA GG-3, antisense 5- TCT TCA CCT GCT CCA CTG CC-3)
- mouse **TNF $\alpha$**  primer (sense 5- AGC CCA CGT CGT AGC AAA CC-3, antisense 5- TAC AAC CCA TCG GCT GGC AC-3)
- mouse **IFN $\gamma$**  primer (sense 5- GCG TCA TTG AAT CAC ACC TG-3, antisense 5- TGA GCT CAT TGA ATG CTT GG-3)
- mouse **IL-1 $\beta$**  primer (sense 5- GGG CTG CTT CCA AAC CTT TG-3, antisense 5- CTG GAA GGT CCA CGG GAA AG-3)
- mouse **GAPDH** primer (sense:5'-CGT CTT CAC CAC CAT GGA GA-3', antisense:5'-CGG CCA TCA CGC CAC AGT TT-3')
- mouse **L32** primer (sense 5- AAG CGA AAC TGG CGG AAA CC-3, antisense 5- CTG GCG TTG GGA TTG GTG AC-3)
- mouse **Foxp3** primer (sense 5- TCC AAT CCC TGC CCT TGA CC-3, antisense 5- CAC ATC ATC GCC CGG TTT CC-3)
- mouse **GATA-3** primer (sense 5- GTC ATC CCT GAG CCA CAT CT-3, antisense 5- AGG GCT CTG CCT CTC TAA CC-3)
- mouse **T-bet** primer (sense 5- CCT GGA CCC AAC TGT CAA CT, antisense 5- AAC TGT GTT CCC GAG GTG TC)

An initial denaturation step for 15 minutes at 95°C, 55 PCR cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 15 seconds were applied. Finally, product homogeneity was verified by melting curve analysis. PCRs were performed using LightCycler Equipment (Roche Company, Indianapolis, USA), and the crossing points were calculated using the second derivative maximum method (included in LightCycler Software) for unknown samples and for DNA standards of known concentrations generated from purified PCR-products of the respective gene. Unknown sample concentrations were calculated according to the standard curve and expressed as percent of average control. Sample

equality or normalization was confirmed by comparable expression of the housekeeping genes GAPDH or L32. After the protocols were established the experiments were replicated two times.

### **3.4.3 TLR and T<sub>H</sub>1/T<sub>H</sub>2 cytokine quantification by LightCycle after in vitro antigens stimulation of trophoblast stem cells (TSCs, SM-10 cell line)**

Placental - Trophoblast Stem Cells (TSCs), SM-10 cell line passaged several weeks until the cell number was sufficient for stimulation. After trypsination followed by washing steps, the cells were counted by CASY TT Cell Counter System, and  $1 \times 10^6$  cells/ml/well cultured in 24-well flat-bottom plates at 37°C in a cell incubator, under 5% CO<sub>2</sub> and 95% humidified atmosphere. Two different protocols was performed, first for  $3.5 \times 10^5$  cells/ml/well and secondly,  $1 \times 10^6$  cells/ml/well. Cells were stimulated with four different allergens, such as Lactobacillus rhamnosus GG (LGG), Lipid A (LA active component of LPS/E.coli), Lipopolysaccharide (LPS/E.coli) and Acinetobacter lwoffii F78 (Ac. lwoffii) at three different concentrations of 10, 50, 100 ng per 1 ml cell suspension, incubation time of 24 and 48h, respectively. The cell-free supernatants were harvested and levels of IL-4, IL-10, IL-1 $\beta$ , TNF $\alpha$ , IFN $\gamma$  were measured using Enzyme-Linked ImmunoSorbent Assay (ELISA). Remaining cells were used in cDNA synthesis. Furthermore, mRNA TLR1 to TLR9, cytokines and chemokines expression (IL-4, IL-10, IL-1 $\beta$ , TNF $\alpha$  and IFN $\gamma$ ) were quantified by LightCycler System as described above.

## **3.5 Protein Analysis**

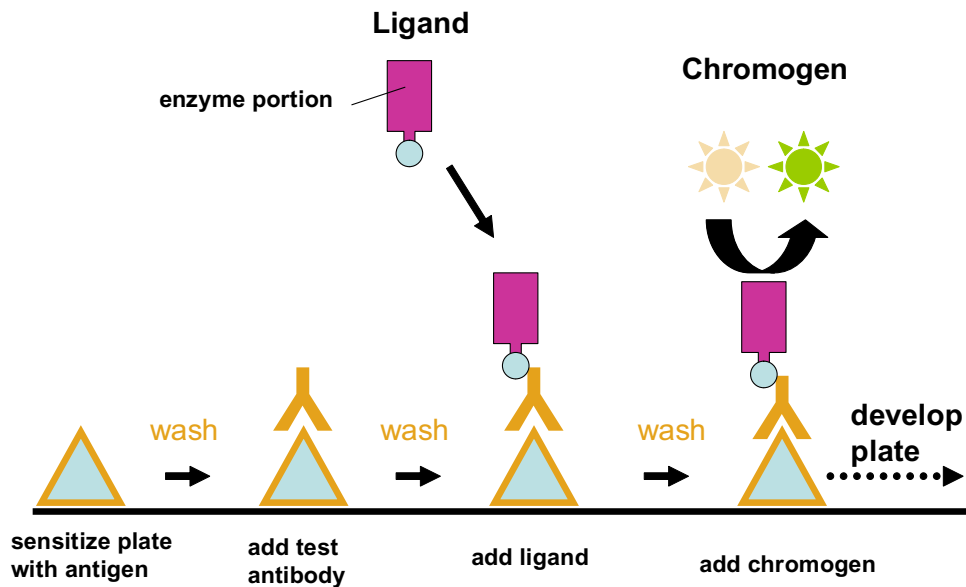
### **3.5.1 Measurement of cytokine and chemokine in bronchoalveolar lavage fluid, amniotic fluid and plasma of un-/pregnant mice by Cytometric Bead Array (CBA)**

Cytometric Bead Array (CBA) is a FACS-based method for analyzing the expression of multiple analytes such as cytokines, chemokines, inflammatory

mediators, immunoglobulines and cell signaling molecules from different biological fluids. Measurement of cytokines and chemokines in bronchoalveolar lavage fluid, amniotic fluid and plasma of un-/pregnant mice, such as IL-4, IL-5, IL-10, IL-12p70, IL-1 $\beta$ , MCP-1, IL-13, TNF $\alpha$ , IFN $\gamma$  were determined by Cytometric Bead Array (CBA; BD Biosciences, San Diego, CA, USA) followed the manufacturer's instruction. There are two commercially available kits: the BD<sup>TM</sup> T<sub>H</sub>1/T<sub>H</sub>2 cytokines Kit (IL-4, IL-5, IL-13, TNF $\alpha$ , IFN $\gamma$ ) and the BD<sup>TM</sup> Inflammation Kit (IL-10, IL-12p70, IL-1 $\beta$ , MCP-1). Different dilutions in range 1:2, 1:4 until 1:4096, in a total volume of 1000  $\mu$ l per tube of cytokine standards were used. Prior the BAL fluid, plasma and amniotic fluid samples were diluted 1:2 and added of 1  $\mu$ l BD Capture Beads Solution (PE-conjugated antibody), in a total volume of 50  $\mu$ l/probe. Both standards and samples were incubated for 1 hour at room temperature (RT) avoid of light. After incubation step, 1 ml of *washing buffer* solution was added in each tube and centrifuged at 200g for 5 minutes. The supernatant was taken off and the remaining sediment of protein was diluted in 300  $\mu$ l washing buffer solution. The samples were analyzed by FACScan /CBA Software - Becton Dickinson Company, USA.

### 3.5.2 Measurement of OVA-specific antibodies in offspring blood serum by Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked ImmunoSorbent Assay (ELISA) is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a *sandwich* ELISA method), (Fig.3.5.2). Thus, levels of OVA-specific IgG1, IgG2a and IgE antibody titers were measured by the ELISA technique. The 96 well microtitre plates (Nunc GmbH, Wiesbaden, Germany) were coated with ovalbumin – OVA - (grade VI; Sigma GmbH, Germany) at 4°C.



**Fig.3.5.2 Enzyme-Linked Immunosorbent Assay (ELISA).** The first antibody binding specific antigen, followed by a covalently binding between antibody and a ligand exposed to specific developing chromogen (according to: Male, D., 2004. Immunology – an illustrated outline. Handbook, the 4<sup>th</sup> Edition, Elsevier Science)

After incubation, plates were rinsed with a washing solution of 0.1% (v/v) Tween 20/PBS and blocked with diluted bovine serum albumin 1% (w/v) BSA/PBS for 2 hours at room temperature. Diluted serum samples and monoclonal OVA-specific IgE (Serotec GmbH, Düsseldorf, Germany), IgG<sub>2a</sub> (Dianova GmbH, Hamburg, Germany) and IgG<sub>1</sub> (Sigma GmbH, Germany) standards are added and the plates are incubated overnight at 4°C. After a new washing step, the plates are coated with biotin-conjugated anti-mouse IgE, IgG<sub>1</sub> or IgG<sub>2a</sub> (BD Biosciences Company, San Diego, USA) and incubated for 2 hours at RT. In the second-step horse-radish peroxidase-conjugated streptavidin (Sigma GmbH, Germany) is added for 30 minutes, at RT, devoid of light. Plates are developed with specific peroxidase substrate (Roche GmbH, Mannheim, Germany). Microtitre plates are *read* by ELISA Reader Program - Magellan3 (Tecan GmbH, Crailsheim, Germany).

### 3.5.3 Measurement of T<sub>H</sub>1/T<sub>H</sub>2 cytokines in offspring bronchoalveolar lavage fluid (BALF) by Enzyme-Linked Immunosorbent Assay (ELISA)

Differential cell counts in bronchoalveolar lavage fluid (BALF) are done after 48h from the last allergen challenge, as describe above. Total leukocytes counts are determined with an automated Casy TT Cell Counter (Schärfe Systems, Reutlingen, Germany). BALF cells were placed in a cytospin and centrifuged at 300g, then stained with May-Grünwald Giemsa staining solution after a prior fixation in Methanol. BALF cell differentiation and counts is performed by light microscopy using standard morphologic criteria. Differential cell counts of 100 leukocytes are performed in duplicates. Remaining BALF volume is centrifuged at 300g and cell-free supernatants were stored in freezer at -20°C for a later measurement of cytokine and/or chemokines levels, such as: IL-4, IL-5, IL-10, IL-13, TNF $\alpha$ , IFN $\gamma$  by using specific antibodies (ELISA). Prior, either the samples are diluted in a range of 1:20 and 1:50 and all first antibodies in a range of 1:250, 1:500, 1:1000 in coating buffer containing 0.1 M NaHCO<sub>3</sub> with pH 8.3. The 96 well microtitre plates (Nunc GmbH, Wiesbaden, Germany) are coated with 50  $\mu$ l/well primary antibody and incubated over night in fridge at 4°C. After incubation the plates are washed with 150  $\mu$ l/well washing solution 0.1% (v/v) Tween 20/PBS using Tecan Columbus Washer System. The plates are blocked with 100  $\mu$ l/well blocking solution 1% (w/v) bovine serum albumin - BSA/PBS for 2 hours at RT. Thus, after a new washing step with the starting concentration of 1000 pg/ml (TNF $\alpha$  and IFN $\gamma$ ) and 500 pg/ml (IL-4, IL-5, IL-10, IL-13) the diluted samples and seven standards, respectively diluted 1:2 are added 50  $\mu$ l/ml. The microplates are incubated 2 hours at RT. After incubation step the plates are washed again, the diluted 1:1000 secondary antibody, a biotin-conjugated anti-mouse is added in a volume of 50  $\mu$ l/well and incubated for 2 hours at RT, on a specific microplates shaker system. In the second-step horse-radish peroxidase-conjugated streptavidin (Sigma GmbH, Germany) is added for 30 minutes, at room temperature devoid of light. Microplates are washed and developed using



100 µl/well BM Blue POD Substrate (Roche GmbH, Mannheim, Germany), for 30 minutes at RT into a dark space. The colour reaction is stopped with the 50 µl/well of stop solution (2 M H<sub>2</sub>SO<sub>4</sub>) and read by ELISA Reader Program - Magellan3 (Tecan GmbH, Crailsheim, Germany), at a wavelength of 450 nm with a reference wavelength of 620nm.

#### **3.5.4 Measurement of T<sub>H</sub>1/T<sub>H</sub>2 cytokines in offspring after in vitro OVA re-stimulation of splenic mononuclear cells (MNCs) by Enzyme-Linked Immunosorbent Assay (ELISA)**

The entire offspring mouse spleen is meshed through a nylon sieve and resuspended into 5 ml of sterile RPMI cell culture medium (RPMI 1640, Cedarlane Company, Hornby, CAN) containing heat-inactivated Fetal Calf Serum 10% (v/v) (FCS), 100 U/ml Peniciline-Streptomycine, 1% (v/v) L-Glutamine. The splenic mononuclear cells were isolated by density gradient centrifugation (Lympholyte<sup>®</sup> M, Cedarlane, Burlington Company, MA, USA). After two washing steps the cells were counted by CASY TT cell counter system and cultured in the 24-well flat-bottom plates in cell incubator under specific parameters (37°C, 5% CO<sub>2</sub> and 95% humidified atmosphere). Thus, 2 x 10<sup>6</sup> cells/ml/well were stimulated with 50 µg/ml OVA in a total volume of 1ml cell suspension. After 72 hours incubation time, cell-free supernatants are harvested and levels of IL-4, IL-5, IL-10, IL-13, TNFα, IFNγ are measured using Enzyme-Linked ImmunoSorbent Assay (ELISA) method.

#### **3.5.5 Measurement of pregnancy steroid hormones in blood serum (BS) and amniotic fluid (AF) of un-/pregnant mice by Enzyme-Linked Immunosorbent Assay (ELISA)**

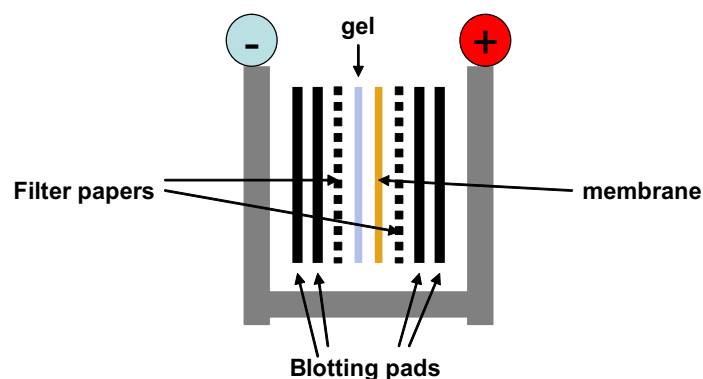
From all supplemented and control groups the steroids hormonal level of estrogen and progesterone (Progesterone human serum-calibrator 50, Beckman Coulter<sup>TM</sup>, USA) in blood serum and amniotic fluid is quantified by ELISA

technique. Following the intrauterine experimental protocol un-/pregnant female mice of Balb/c and C57BL/6 strains are supplemented with LGG, Ac. Iwoffii F78, LA and control animals with PBS and incubated for 10 minutes at 55°C. The blood was collected from tail in sterile condition, before mice were euthanized at pregnancy term (day 18) and preserve for 60 minutes in fridge at 4°C followed by a centrifuged step with 4000 rpm, for 20 minutes at RT. Collecting amniotic fluid, the pregnant mice are euthanized, the uterus was opened and a volume of 50-1000 µl had been pulling out with a sterile syringe. Either, serum or amniotic fluid can be preserved for later analysis in freezer, at -20°C for one month.

### **3.5.6 Western Blot Analysis (WB): TLR detection in maternal organs**

Western blot (WB) is a method to detect a specific protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (under denaturing condition) or by the 3-D structure of the protein (native/non-denaturing conditions). After cell or tissue extraction the proteins are then transferred to a special membrane (Nitrocellulose or Poly-1,1-difluoroethene or polyvinylidene difluoride - PVDF), where they are detected using specific antibodies (monoclonal and/or polyclonal antibodies) to the target protein. This has dramatically reduced the time to carry out a blot. Maternal organs such as small intestine, spleen and placenta are collected into liquid nitrogen, preserved and stored in freezer at -80°C. For protein extraction the thawing process is initiated using a new protocol. TriZol lysing buffer 1 mL is used for 50 mg organ (as well as  $\leq 5 \times 10^6$  cells/ml culture medium) to lyses the cells, respective to destroy both connective tissue and membrane cells, in order to release the entire amount of proteins. The separation phase step containing RNA (transparent supernatant, SN) and proteins tissue mixture (the sediment) is performed using 200 µl of Chloroforme followed by cold centrifuged under 4°C temperature, for 15 minutes at 12,000g. Thus, the supernatant containing isolated total RNA could be preserved in absolute EtOH, in freezer under -80°C, whilst sediment containing total amount of proteins is

treated with Isopropanol for protein extraction. A new centrifugation is performed for 10-15 minutes under 4°C at 12,000g. The protein pellet is treated with 2 ml Guanidinehydrochlorid 0.3 M in Ethanol 95% (v/v) followed by ultrasonification for 10 minutes. This extraction process is performed twice and followed by a protein washing step with absolut EtOH. By incubation step the protein pellet is denaturated by 40 µl Lämmli buffer, for 5 min at 95°C, followed by a new centrifuged 10-15 minutes under 4°C at 12,000g. The samples are preserved in freezer under -80°C. After the protein extraction the concentration is measured using the BCA Kit a protein concentration measurement method by the manufacturer's intructions. Absorbtion curve is completed by Tecan Reader Program – Magellan 3 at the wave leght of 562 nm. Previously, the *SDS dispersive gel* and *collector gel* are prepared and the spacers are loaded with 25 µl of mixture solution containing protein and sample buffer (diluted 30:1) and protein marker (MagicMark™ XP Western Protein Standard). The proteins negatively charged by SDS, leave themselves from an uncolored Polyacrylamidgel on a Nitrocellulose membrane after the *Semydry buffer* is applied. Methanol a potent activator of the membrane is applied before the protein transfer. Thus, the TLR2 and TLR4 target proteins are transferred for 1h at 65V to a membrane of Nitrocellulose or PVDF the blot equipment which consists of two graphite electrodes (Fig. 3.5.6).

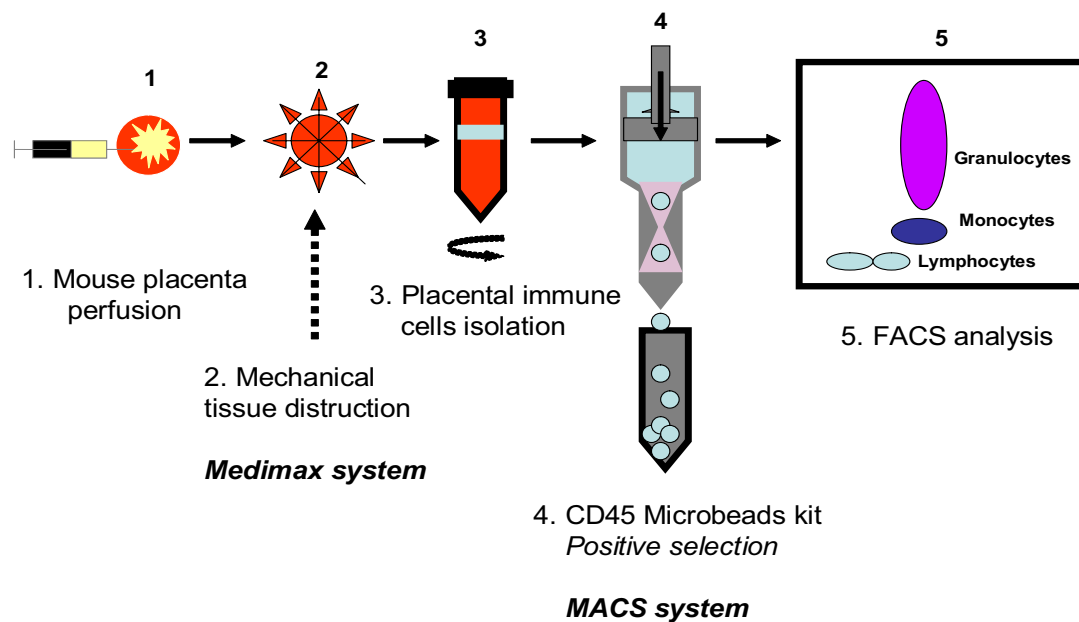


**Fig.3.5.6 Western Blot Analysis (WB)** – the target proteins transfer process from SDS gel to a Nitrocellulose or PVDF membrane was utilized to express TLR in placental, gut or spleen maternal tissues.

The protein transfer is verified by Ponceau staining before the antibodies are added. The membrane is washed with distilled water and blocked by a blocking solution of Milk 3% (wt/v) reconstituted in PBS, for 30 min. The target proteins are detected in two steps for 1 hour at RT, roller-shacked. As first antibody rabbit anti-mouse TLR2 and TLR4 monoclonal or polyclonal antibodies diluted into milk solution (diluted 1:400) are used and as secondary antibody an anti-rabbit-goat HRP-conjugated (diluted 1:2000), respectively. After the antibodies are applied, the membrane is washed twice in TTBS. The development step is done by Picco Developer Kit/Femto solution (Pierce Company, USA), and dry with non-porously Whatmann papers. A Röntgen Polymer Film was laid over the membrane being closed into a black box and avoiding light at different time-points, in order to become high quality images. Because the detection of targeted proteins by WB is very sensitive method, specific antibodies, fresh made gels and buffers is mandatory to be done before to start.

### **3.5.7 Magnetic-Absorbed Cell Sorting (MACS): positive selection of total CD45<sup>+</sup> cells from murine placenta**

Magnetic-activated cell sorting (MACS) is a method used for separation of various cell populations depending on their surface antigens (CD molecules). The term MACS is a registered trademark of Miltenyi Biotec. Since fluorophore-conjugated antibodies are much more prevalent, it is possible to use magnetic beads coated with anti-fluorochrome antibodies. They are incubated with the fluorescent-labelled antibodies against the antigen of interest and may serve for cell separation with respect to the antigen. Thus, the separated mixture of cells is incubated with magnetic beads solution. Afterwards, the cell solution is transferred on a column placed in a strong magnetic field. In this step, the cells attached to the beads (expressing the antigen) stay on the column, while other cells (not expressing the antigen) flow through.



**Fig.3.5.7 Placental immune cells isolation by positive selection - MACS System and characterisation by Flow Cytometry System.** The experimental protocol includes four other protocols of work, such as: (1) mouse placenta perfusion; (2) mechanical tissue destruction; (3) placental immune cells isolation; (4) positive selection; (5) placental cell characterisation.

By this method, the cells can be separated positively or negatively with respect to the particular antigen(s). Removing the MACS column from the magnetic field a positive selection the cells expressing the antigen of interest, which attached to the magnetic column are washed out into a separate glass vessel.

The general experimental protocol of placental immune cells isolation includes five other protocols of work applied exclusively for placental tissue in animal model, such as: (1) mouse placenta perfusion – after a prior mouse's anaesthesia, a total perfusion of the mouse is needed (through the left ventricle of the heart, using a sterile 10 cc syringe and 4-8 ml saline solution NaCl 0.9% (v/v), with a constant pressure solution); then, a local placenta perfusion is performed; (2) mechanical tissue destruction by a Cell-Shredder (79400S Medicons, 50  $\mu\text{m}$ , DAKO Cytomation, Hamburg, DK) and DAKO Medimachine (DAKO Cytomation, Hamburger, DK); the placenta pieces are placed into a steel mesh with 50  $\mu\text{m}$  pore diameter grated, followed by five times rinse with cold

PBS; thus the placenta cell suspension obtained is stored immediately in ice; (3) placental immune cells isolation with Lympholyte M, Percoll<sup>®</sup>/PAN-COLL mouse or Ficoll Isopaque gradient solutions (usually to isolate cells of different densities and in particular, to purified the lymphocytes). The diluted sample of cell suspension added to the gradient solution and centrifuged. Because red blood cells and polymorphs remained into cell suspension after perfusion they sediment to the bottom of tube, while the lymphocytes and some macrophages remain at the interface. Lymphocyte population may be further depleted of macrophages by adherence or by letting the phagocytes take up iron filings and then removing them with a magnetic field system), and (4) positive selection by MACS method using the CD45 Isolation Kit (Miltenyi Biotec, Germany). Prior to cell suspension load onto a MACS<sup>®</sup> Column (placed into a strong magnetic field of a MACS Separator), the CD45<sup>+</sup> cells are magnetically labelled with CD45 Microbeads and incubated at 4°C for 20 min, avoid light. The magnetically labelled CD45<sup>+</sup> cells are retained on the column. The unlabeled cells run through and this cell fraction is depleted of CD45<sup>+</sup> cells. After column removal from the magnetic field, the magnetically retained CD45<sup>+</sup> cells can be eluted as the positively selected cell fraction. The 5<sup>th</sup> experimental protocol includes placental cell characterisation using an extra-cellular staining protocol and capturing the cell population images by a sorting instruments, like FACS (fluorescent-activated cell sorting system). This method is useful for isolation of a particular cell type. Magnetic beads conjugated to an antibody against an antigen of interest are not always available, but there is a way to circumvent it.

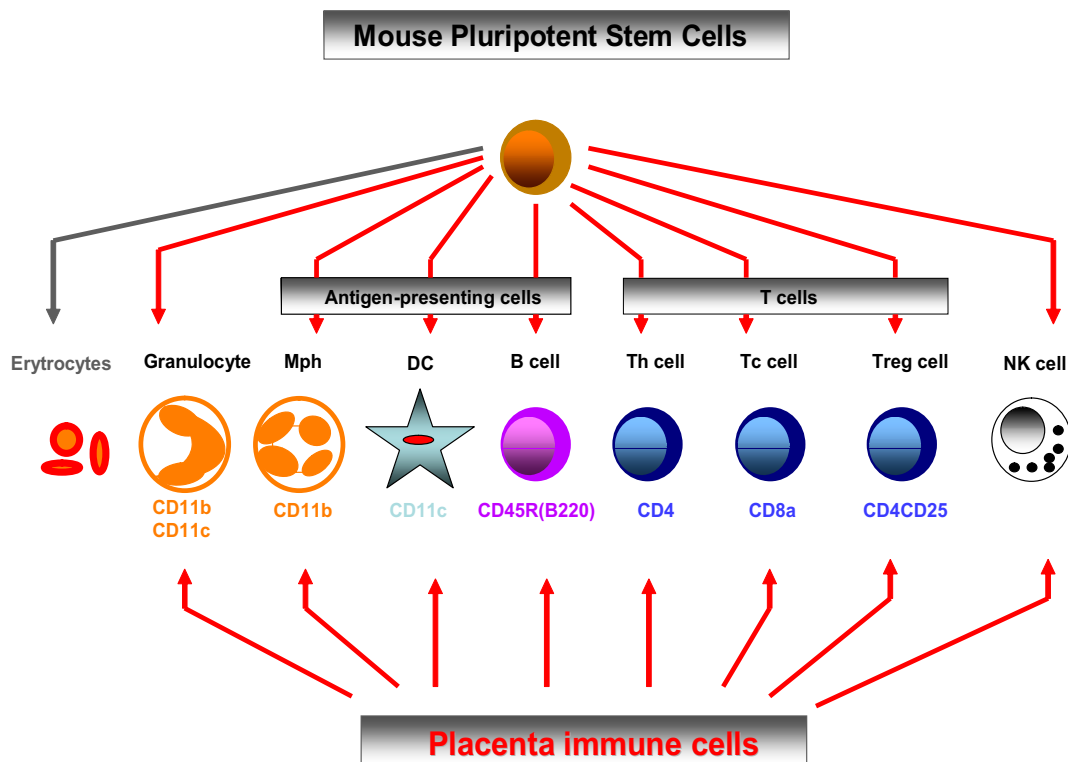
### **3.5.8 Characterisation of placental immune cells by Flow Cytometry (FACS)**

Subsequent to MACS positive selection of total CD45<sup>+</sup> lymphocytes of placental tissue the cell suspension is centrifuged at 350g, for 10 minutes then washed with sterile PBS, and the pellet resuspended in 1 ml FACS buffer. The cell number is counted and the suspension volume is split in four tubes. FACS tube

contains  $1 \times 10^6$  cells/333  $\mu$ l FACS buffer, then centrifuged at 350g for 10 minutes (using centrifuge brakes). After centrifugation the cell pellet is resuspend in 1  $\mu$ l mice serum and 9  $\mu$ l FACS buffer. Additionally, the placental immune cells are harvested and stained with fluorochrome-conjugated 1  $\mu$ l mouse-Abs and rat-Abs as isotype control (Fig./Tab.3.5.8).

Tubes	1	2	3	4	Isotype control
FITC	CD 3	CD 25	NK 1.1	CD 11c	Rat IgM,k
PE	CD 8	CD 69	B 220	CD 11b	Rat IgG2a,k
PerCP	C D 4	CD 4	CD 3	CD 3	Rat IgG2a,k
APC	CD 45	CD 45	CD 45	CD45	Rat IgG2b,k

**Tab.3.5.8 Experimental protocol of placental immune cells.** An extracellular staining method developed exclusively for FACS analysis



**Fig.3.5.8 Schematically illustration of placental immune cells and specific markers** (according to: MACS<sup>®</sup> Animal Cell Isolation Scheme – The optimal solution for cell isolation in immunological research, Miltenyi Biotec GmbH, 2007)

Incubation step is performed at room temperature, 20 minutes avoid of light. Then, the cell pellet is washed out in 1 mL FACS buffer and centrifuged at 350g, for 10 minutes and resuspend in 300 µl Cell-fix solution of 1% (v/v) (dilution of 1:10 of 4% formaldehyde reconstituted in distilled water). Using appropriate laser detectors, they send to the cells characteristic light signals in followed range of the wavelength of light emissions: 515-545 nm/564-606 nm/650 nm. The system is setting for the four colors analysis of cell surface antigens, such as: FITC, PE, Per CP and APC marks. The FACS sorting system consists of the Flow Cytometer as a sensor unit, a computer workstation and various software programs. Placental immune cells were analyzed by means of flow cytometry on a FACScan by using the CellQuest 3.22i Software (Becton Dickinson Company and Apple McIntosh PC, Franklin Lake, USA).

#### **3.5.9 Offspring lung and maternal small intestine, spleen and placenta histopathology: Haematoxylin & Eosin (HE) and Periodic acid-Schiff (PAS) staining methods**

Previously, the organs were perfusing with PBS buffer, pH 7.2 and in situ fixed with formaldehyde 4% (wt/vl) solution. Entire tissues were paraffin-embedded and 3 µm sections cut by Microtome. Tissue histopathology including the tissue structural alter, inflammatory cells accumulation and mucus production were observed with Haematoxylin/Eosin (H&E) and Periodic acid-Schiff (PAS) stained sections using high-power light microscopy. Paraffin-embedded sections like maternal small intestine, spleen and placenta respective offspring lung are deparaffinized and rehydrated in xylene 2 x 15 minutes, respectively 2 x 5 minutes in a descending concentration of ethanol (100%, 90%, 80%, 70% and 50%) (v/v). Thus, the samples are prepared for the staining steps in Haematoxylin solution/Gill III - 10 minutes and Eosin solution – 5 minutes. Following staining steps, tissue slides are fixed with an ascending series of ethanol (70%, 80%, 90% and 100%), 1 x 1 minute and into Neo-clear solution or Xylol, 1 x 15minutes. The slides are counterstained with Neo-Mount or Entellan-



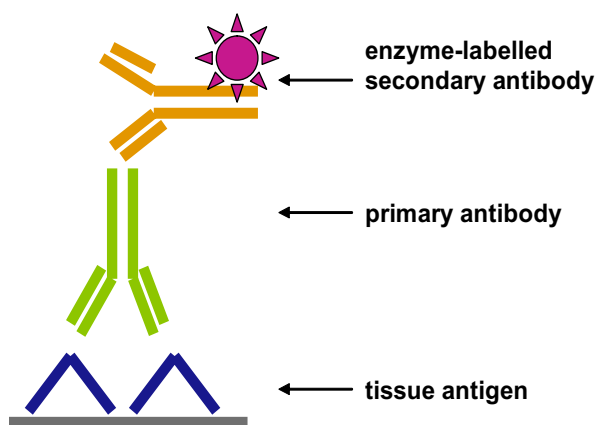
New. *Haematoxylin/Eosin (H&E)* is a qualitative analysis of histological preparations. The staining procedure allows the differentiation of various cells using morphological criteria. The system uses two different colors specific for the basophile/azurophile - *Haematoxylin* (blue staining) and the acidophil/eosin - *Eosin G.* (red staining). The *Haematoxylin* binds mainly to positively charged proteins, such as histones in the cell nuclei (hence turn the cell nucleus blue), while *Eosin* binds to negatively charged proteins from the target cell or rest of the tissue.

The reaction of Periodic acid selectively oxidizes the glucose residues, resulting aldehydes that react with the Schiff reagent and creates a purple color. PAS staining is mainly used for staining structures containing a high proportion of carbohydrate macromolecules such as: glycogen, glycoprotein, proteoglycans typically found in connective tissues, mucus, and basal lamina. Deparaffinization and rehydration steps are identical as in H&E staining. Thus, the sections are bathed 1 x 5 minutes in Periodic acid and 1 x 15 minutes Schiff reagent. Again, the stained tissue slides were fixed through an ascending series of ethanol (70%, 80%, 90%, 100%), 1 x 1 minutes each, then into Neo-clear or Xylol, 1 x 15 minutes. The slides are counterstained with *Neo-Mount* or *Entellan New*. Both H&E and PAS stained tissue slides are examined under a Light microscope (Olympus BX51<sup>®</sup>).

### 3.5.10 Immunohistochemistry (IHC) of maternal main investigated organs

Immunohistochemistry (IHC) refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. "Two-step Indirect Immunohistochemistry" is used to stain CD4<sup>+</sup> T cells, TLR2 and TLR4 expressed by APC- and Trophoblast cells by Vector<sup>®</sup> M.O.M.<sup>™</sup> Immunodetection Kit for both HPR (Horseradish Peroxidase) or AP (Alkaline Phosphatase) systems. The indirect method of immunohistochemical staining involves an unlabeled primary antibody, for a specific tissue antigen, and a labeled secondary antibody, which binds the

primary antibody (the secondary antibody must be against the IgG of the animal species in which the primary antibody has been raised). The method is very sensitive due to signal amplification, through several secondary antibody reactions with different antigenic sites on the primary antibody. Commonly, the biotinylated secondary antibody is coupled with Streptavidin-Horseradish Peroxidase (S-HRP) or Streptavidin-Alkaline Phosphatase (S-AP). This reacts with 3, 3'-Diaminobenzidine (DAB) in order to produce a brown staining. The primary and secondary antibodies are attached in this procedure known as DAB staining. The reaction can be enhanced using Nickel (Ni), producing a deep purple-gray staining at the end for HRP system. In case of AP system the specificity of the staining is blue (Fig.3.5.10).



**Fig.3.5.10 Two-step indirect immunohistochemistry** – enzyme-labelled secondary antibody reacts with primary antibody bound to tissue antigen (according to: Boenisch, T., et al., 2001. Immunohistological staining methods. Handbook the 3<sup>rd</sup> Edition, Dako Cytomation, Carpinteria, California, USA)

To obtain the tissue slides, a microtome (Accu-Cut<sup>®</sup> SRM<sup>™</sup> 200 Rotary microtome, Sakura Finetek, USA Inc.) is applied. It is a mechanical instrument used to cut biological specimens into very thin segments for microscopic examination. Most microtomes use a steel blade for cutting animal tissues in very fine histological sections. The most common applications of microtomes used are: (i) traditional histological technique: tissues are hardened by replacing water with paraffin. The tissue is then cut in the microtome varying from 2 to 25 µm thickness. Thus, tissue is mounted on a microscope slide stained and examined using a light microscope (LM) or fluorescent microscope (FM); (ii) cryosections: water-rich tissues are hardened by freezing and cut frozen; sections are specific

stained and examined by a light microscope. Accordingly, in our study maternal paraffine-embedded tissues like small intestine, spleen and placenta as well as offspring lung are used. The 3 µm sections are deparaffinized and rehydrated performing steps described at H&E/PAS staining. Moreover, the maternal cryo-embedded tissues of 10 µm cryosections, are rehydrated in Xylene for 2 x 15 minutes and different concentrations of ethanol (100%, 90%, 80%, 70% and 50%) for 2 x 5 minutes. Sections are rinsed in tap water for 5 minutes and the antigen retrieval applied by boiling the sections into microwave using a 10 mM Citrate buffer. The sections are immediately cool down in ice for 20 minutes. The cryosections are dried in air for 10 minutes and fixed in cold acetone for 10 minutes. The endogenous peroxidase activity is blocked with a blocking solution of 5% goat- or rabbit serum anti-mouse IgG reconstituted in sterile 1x PBS for 5 minutes. The enzymatical activity can be inhibiting by a mixture of sodium azide and H<sub>2</sub>O<sub>2</sub>. The sections are rinsed in PBS and the first antibody (1:100), such as polyclonal rabbit anti-mouse TLR2, TLR4 or CD4 T cells applied, for 2 hours of incubation time. Afterwards, the secondary antibody - biotinylated goat anti-rabbit (1:200) is applied for 1 hour of incubation time. Using light microscopy the sections are covered with undiluted streptavidin – peroxidase-conjugated reagent for 15 minutes. Fluorescence microscopy requested a streptavidin – FITC-conjugated reagent for 5 minutes. The peroxidase substrate of 0.1% DAB - 0.3% H<sub>2</sub>O<sub>2</sub> (v/v) is applied for 10 minutes, increasing the staining procedure intensity. The counterstaining is performed using a Mayer Hematoxylin staining solution, for 6-8 minutes or Fluorescein depending on the preferred microscopy. Then, the sections are "blue" with a solution of 1% NaOH (v/v) reconstituted in distilated water, pH 9.5-9.8, for 3 minutes. The preceding paraffine sections passed backward through ethanol baths and xylol to fix the staining (as described at H&E/PAS staining). The stained tissues are covered with colorless nail polish or other alcohol insoluble covering media (Histomount mounting solution, Zymed Laboratories, San Francisco, USA) and studied under the light microscope, respective fluorescent microscope (Olympus BX51<sup>®</sup>, Olympus Deutschland GmbH), using particular filters.

**3.5.11 Electron microscopy of placenta after prenatal supplementation**

After embedding tissues in Epoxy resin, a microtome equipped with a glass or diamond knife is used to cut very thin sections (typically 60 to 100 nanometers). Sections are stained and examined with a transmission electron microscope. This instrument is often called an ultramicrotome. Placenta samples were fixed by immersion into a mixture of 1% glutardialdehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 2 hours in the cold PBS. After rinsing with 0.1 M sodium cacodylate buffer, samples are post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hours, rinsed in the same buffer for 4 x 5 minutes, washed in distilled water for 2 x 5 minutes, and stained on-bloc in half-saturated uranyl acetate over night. After rinsing with distilled water 4 x 5 minutes, samples are dehydrated through an ascending series of acetone (70%, 90%, 100%) for 2 x 10 minutes, transferred into a 1:1 mixture of acetone and Araldite for 1 hour and finally, into pure Araldite, over night. After transfer into fresh resin, samples are polymerised at 60°C for three days. Ultrathin sections are cut on an Ultracut-E® (Reichert-Jung, Wien, Austria), collected on coated nickel grids, stained with lead citrate and uranyl acetate, and analysed using a Zeiss EM 900 (Zeiss®, Oberkochen, Germany) (according to: Fehrenbach, H, 2007).

**0.2 M Cacodylate buffer - stock solution**

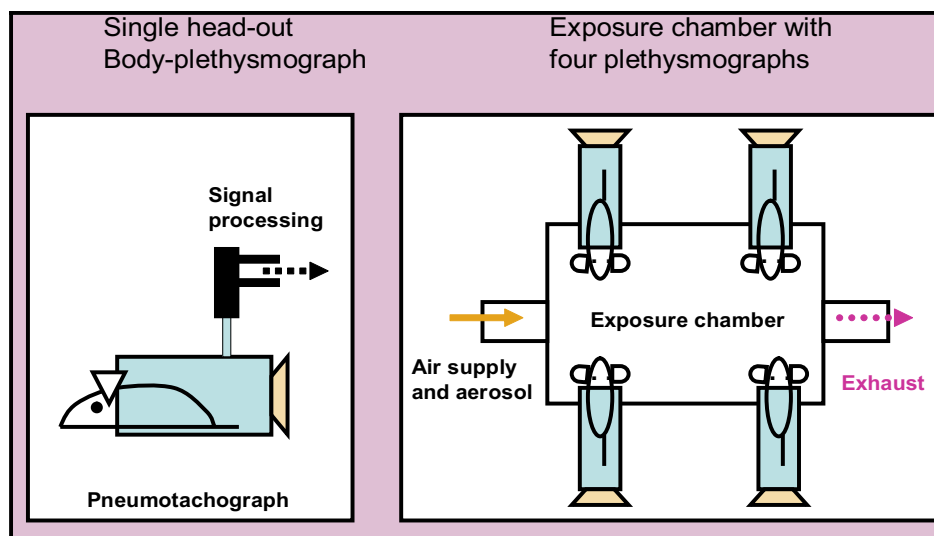
250 ml distilled H<sub>2</sub>O  
20.15 g sodium cacodylate trihydrate (MW = 214)  
0.1 ml HCl  
pH<sub>final</sub> 7.4

**0.1 M Cacodylate buffer, solution with 3% sucrose and 0.1% CaCl<sub>2</sub>**

30 g RNAase-free sucrose (can use regular sucrose if not making it for EM)  
0.05 g CaCl<sub>2</sub> or 1 ml of a 1M CaCl<sub>2</sub> stock solution  
250 ml 0.2 M cacodylate stock solution  
250 ml distilled H<sub>2</sub>O

### 3.6 Lung function analysis. Measurement of Airwayhyperreactivity (AHR) to $\beta$ -Methyl-Acetylcholine (MCh) using Head-out-body-plethysmography

Changes in lung functions were determined by noninvasive head-out body plethysmography. The development of acute airway obstruction after OVA challenge and airwayhyperreactivity (AHR) after methacholine aerosol application (after 24 hours from last challenge) were assessed by a decrease in the mid-expiratory flow rate ( $EF_{50}$ ). The Head-out Body Plethysmograph (HOBP) system is a glass made cylinder-like attached to aerosol exposure chamber (Forschungsstätten Medical School Hannover, Germany). Each mouse is positioned in the head-out body plethysmograph while the head of the animal protuded through a neck collar (9 mm inner side diameter; RoekoLangenau, Germany) into the aerosol exposure chamber which is ventilated by continuous airflow of 200 ml/min.



**Fig.3.6 Offspring lung function analysis.** Schematically illustration of measurement of airway hyperreactivity (AHR) to  $\beta$ -Methyl-Acetyl-Choline (MCh) by Head-out-body-plethysmography (according to: Wegmann, M., Animal Models of Experimental Asthma. In Animal Models of T Cell Mediated Skin Diseases. Erst Schering Res Found Workshop. Axel Springer Verlag 2004(50): 69-87)

For airflow measurement, a calibrated Pneumotachograph (PTM 378/1.2; Hugo Sachs Elektronik, March-Hugstetten, Germany) and a differential Pressure Transducer (8T-2; Gaeltec, Dunvegan, Scotland) coupled to an Amplifier (HSE-IA; Hugo Sachs Elektronik) are attached to the top port of each plethysmograph. For each animal, the amplified analog signal from the pressure transducer was digitized via an Analog-Digital Converter (DT301 PCI; Data Translation Marlboro, MA). For data calculation Notocord hem 3.5 Software (Notocord®, Paris, France) is used. During continuous assessment of EF<sub>50</sub>, mice were exposed to  $\beta$ -Acetyl-Methacholine (MCh) aerosols with rising concentrations (12.5, 25, 50, 75, 100 or 125 mg/ml), and airway hyperreactivity was expressed as the concentration of MCh that caused a 50% reduction in baseline mid-expiratory airflow (MCh mg/ml) using subsequently parameters:

15 `baseline measurement  
15 `10 `` PBS  
19 `50 `` - 12.5 mg/ml MCh  
24 `30 `` - 25.0 mg/ml MCh  
29 `10 `` - 50.0 mg/ml MCh  
33 `50 `` - 75.0 mg/ml MCh  
38 `20 `` - 100 mg/ml MCh  
43 `10 `` - 125 mg/ml MCh  
46 `40 `` end of measurement

The  $\beta$ -Acetyl-Methacholine (MCh) is a derivative of the neurotransmitter acetylcholine, with potential action transmission in the neurons and neuromuscular synapse. The animals tested inhaled the MCh aerosol that binds into to the cholinergic receptors the neuromuscular synapse, and give a signal to muscle contraction. However, the effect is only locally on the smooth respiratory muscles. Therefore, the resulting bronchial obstruction shows an increase in respiratory resistance. This means that each animal have to breathe against an increase in respiratory resistance, leading to a slow down expiration, meaning a reduced expiratory respiratory flow.

In animals with an allergic inflammation in the lungs is an increased sensitivity to non-specific stimuli like MCh, thus those animals may respond significantly to lower concentrations of MCh with a 50% reduction in the  $EF_{50}$ . Diversity of values depends on the species used. They are modified by the genome of the respective animal, so it is necessary to measure for any attempt an appropriate number of appropriate controls - depends on the experimental protocol and the species used.

### 3.7 Statistics

Analyses done with Microsoft Excel as well with One-way ANOVA or Student's unpaired t -test (GraphPad Prism<sup>®</sup> Software, version 4.01) to determine the level of significance between the treated and untreated animal groups. All numerical data expressed  $MEAN \pm S.E.M$  (Standard error of the mean value). Results are considered statistically significant for  $p < 0.05$ .

### 3.8 Materials

Consumables	Provider
Centrifuge tubes, sterile (15 ml, 50 ml)	VWR GmbH, Germany
CASYcup with lid	Innovatis AG, Bielefeld, Germany
FACS tubes without lid	Sarstedt GmbH, Nümbrecht, 551579, Germany
Plasma tubes (Microvette #500K3E, contain Tri-K-EDTA)	Sarstedt GmbH, Nümbrecht, 551579, Germany
ELISA 96-well plates	Nunc Maxisorp, NUNC, 437958, Germany
Cell culture 24-well plates	Nunc Maxisorp, NUNC, 142475, Germany
Cell culture bottles (25cm <sup>2</sup> )	Nunc Maxisorp, NUNC, 156367, Germany

Cell culture bottles (75cm <sup>2</sup> )	Nunc Maxisorp, NUNC, 156499, Germany
Perfusion butterfly needle	BD Bioscience, Heidelberg, Germany
Syringes and needles for injection	BD Bioscience, Heidelberg, Germany
Nylon Cell sieves (100µm)	Falcon BD, Heidelberg, 352360, Germany
Syringe-Fitting, 50µm	CN01330, 79400 Medicons Dako GmbH, Germany
Filter paper	Whatmann, Maidstone, UK
Glass cover slips(24 x 50mm <sup>2</sup> )	Menzel-Glass, Germany
Cryotubes(2 ml)	Sarstedt GmbH, Nümbrecht, 72694106, Germany
Filters (0.22 µm and 0.45 µm)	Kobe Lab.& Intsr., Marburg, FA0371-1, Germany
LC capillary	Roche GmbH, 1909339, Germany
Hand gloves (S,M size)	NOBA GmbH & Co., 905451/2, Germany
Hyperbond Nitrocellulose Membrane	Amersham GmbH, 25012345, Germany
DF-Membrane	Millipore GmbH, IPVH 00010, Germany
<b>Antibodies, Enzymes and Kits</b>	<b>Provider</b>
Ovalbumin chicken Egg Grade VI (OVA)	Sigma GmbH, A-2512, Germany
Monoclonal Anti-Chicken Egg Albumin(Clone Ova-14)	Sigma GmH, A-6075, Germany
Mouse Serum	BioMedizinisches Forschung Zentrum, Marburg, Germany
dNTP Mix 10 mMol	Invitrogen GmbH, 18427-088, Germany



RNA Later	Qiagen GmbH, Hilden, Germany
RNA Sample Buffer	Sigma GmH, r4268, Germany
DNA 100bp plus 100-3000bp	Peqlab GmbH, Germany
DNA 50bd -Leiter	Peqlab GmbH, Germany
DNA Leiter High Range Mix	Peqlab GmbH, Germany
BD™ Cytometric Bead Array(CBA) Kit	BD Biosciences, San Diego, CA, USA
RNeasy Mini Kit(50)	Qiagen GmbH, Hilden, Germany
Rneasy Lipid Tissue Mini Kit(50)	Qiagen GmbH, Hilden, Germany
DNA-free Set	Ambion, Austin, TX, USA
RNase-free DNase Set	Qiagen GmbH, Hilden, Germany
Rneasy Cleanup Mini Kit (50)	Qiagen GmbH, Hilden, Germany
Omniscript RT Kit(200)	Qiagen GmbH, Hilden, Germany
Avidin Biotin Blocking Kit	Vector Lab. USA, sp-2001
CBA mouse inflammation Kit	BD Bioscience, Heidelberg
HotStart Taq Master Mi Kit	Qiagen GmbH, Hilden, Germany
MOM Basic Kit	Vector Laboratories, USA
MOM Fluorecein Kit	Vector Laboratories, FMK-2201, USA
PAS Staining Kit	Merk GmbH, Germany
Quanti Tect SyberGreen® PCR Kit	Qiagen GmbH, Hilden, Germany
<b>Antibodies used in IHC</b>	<b>Provider</b>
Purified anti-mouse CD4(L3T4)	BD Pharmingen, Heidelberg, 550280, Germany
Purified rabbit anti-mouse TLR2 (clone 2.5 mT2)	Technical University of München, Germany
Purified rabbit anti-mouse TLR4/MD2 (clone MT S510)	eBioscience, Heidelberg, Germany
Goat anti-rabbit IgG polyclonal biotin.	BD Bioscience, Heidelberg, Germany
<b>OVA specific Antibodies for ELISA</b>	<b>Provider</b>
Anti-mouse IgE biotinylated	BD Pharmingen, 553419, Heidelberg, Germany

Anti-mouse IgG1 biotinylated	BD Pharmingen, 553441, Heidelberg, Germany
Anti-mouse IgG2a biotinylated	BD Pharmingen, 553388, Heidelberg, Germany
<b>Antibodies used in WB</b>	<b>Provider</b>
Rabbit serum anti-mouseTLR2 polyclonal(clone 2.7 mT2)	Technical University of München, Germany
Rabbit serum anti-mouseTLR4 polyclon.	eBioscience, 24900491, Heidelberg, Germany
<b>Antibodies used in BD™ Cytometric Bead Array Kit</b>	<b>Provider</b>
Interleukin-4	CBA Kit 558298, BD Biosciences, San Diego, CA, USA
Interleukin-5	CBA Kit 558302, BD Biosciences, San Diego, CA, USA
Interleukin-10	CBA Kit 558300, BD Biosciences, San Diego, CA, USA
Interleukin-12p70	CBA Kit 558303, BD Biosciences, San Diego, CA, USA
Macrophage Chemotactic Protein-1(MCP-1)	CBA Kit 558342, BD Biosciences, San Diego, CA, USA
Tumor Necrosis Factor- $\alpha$	CBA Kit 558299, BD Biosciences, San Diego, CA, USA
Interferon- $\gamma$	CBA Kit 558296, BD Biosciences, San Diego, CA, USA
<b>Antibodies used in FACS analysis</b>	<b>Provider</b>
Rat IgM,k – FITC	BD Pharmigen, USA
Rat IgG2a,k – PE	BD Pharmigen, 553930, USA

Rat IgG2a,k – PerCP	BD Biosciences, 553933, San Diego, CA, USA
Rat IgG2b,k – APC	BD Biosciences, 553991, San Diego, CA, USA
Rat anti-mouse CD3e - FITC	BD Biosciences, 01084D, San Diego, CA, USA
Rat anti-mouse CD25 - FITC	BD Pharmigen, 553071, USA
Rat anti-mouse Pan-NK cells - FITC	BD Biosciences, 09944D, San Diego, CA, USA
Rat anti-mouse CD11c - FITC	BD Pharmigen, USA
Rat anti-mouse CD8a - PE	BD Pharmigen, 553033, USA
Rat anti-mouse CD 69 - PE	BD Pharmigen, 01505B, USA
Rat anti-mouse CD45R/B220 - PE	BD Biosciences, 553089, San Diego, CA, USA
Rat anti-mouse CD11b - PE	BD Pharmigen, M041984, USA
Rat anti-mouse CD4 - PerCP	BD Pharmigen, 553052, USA
Rat anti-mouse CD3e – PerCP	BD Pharmigen, USA
Rat anti-mouse CD45 - APC	BD Pharmigen, 559864, USA
<b>Anaesthetics</b>	<b>Provider</b>
Isoflurane	Abbott, Wiesbaden, Germany
Ketanest 50mg/ml	Fa.Parke-Davis, USA
Ketaminhydrochlorid	PZN 7810239, Reg.Nr.K693, Germany
Rompun 2% injection solution	Fa.Bayer, Germany
Xylazinhydrochloride	PZN 1320422, Reg.Nr.R1061, Germany
<b>Reagents</b>	<b>Provider</b>
0.9% NaCl sol., physiological	B.Braun AG, 7275A251, Melsungen, Germany
Al(OH) <sub>3</sub>	Pierce Company, 77161, Illinois, USA,

1x Trypsin EDTA	PAA Laboratories GmbH, Germany
10x Trypsin EDTA	PAA Laboratories GmbH, Germany
RPMI 1640 without L-Glutamine	Cambrex, East Rutherford, NJ
Penicilline -Streptomycine	PAA Laboratories GmbH, Germany
Actinomycine	PAA Laboratories GmbH, Germany
L-Glutamine	Gibco GmbH, 25030-024, Germany
Na Pyruvat 100mM	PAA Laboratories GmbH,s11-003, Germany
Na <sub>2</sub> CO <sub>3</sub> powder	Merk, A 407992, Germany
Fetal Calf Serum Gold(FCS)	Biochrom AG, Berlin, Germany
Bovines Serum Albumin (BSA)	Sigma GmbH, A-9647, Germany
Sulphuric Acid(H <sub>2</sub> SO <sub>4</sub> )	Merck GmbH,1.00731.1011, Germany
Washing solution POD	Roche GmbH, 1484281, Germany
Tween 20	Merk GmbH, 822184, Germany
Phosphat buffered saline solution (PBS)	PAA H15002, Austria
Phosphat buffered saline powder(PBS)	Biochrom GmbH, L182-50, Germany
NaHCO <sub>3</sub>	Merk GmbH, 6329, Germany
Isopropanol	Sigma GmbH, Germany
Chloroform	Roth GmbH, 3313.4, Germany
TriFast lysis buffer	PeqLab GmbH, 30-2020, Germany
TRIzol <sup>®</sup> Reagent	Molecular Research Center Inc., Cincinnati, OH, USA
TAE running buffer	Sigma GmbH, Germany
TBE running buffer	Sigma GmbH, Germany
RNA - Agarose powder	Sigma GmbH, Germany
DNA – Agarose powder	Sigma GmbH, Germany
Sample Loading Buffer	Sigma GmbH, Germany
RNA Ladder(100bp)	Sigma GmbH, Germany

Liquid Nitrogen	Chemistry Institute of Marburg, Germany
Tissue Tek	Sakura, Zoeterwoude, NJ
Streptavidine-HRP	Sigma GmbH, S 5512, Germany
Streptavidine-AP	Sigma GmbH, Germany
Ethanol absolute	Riedel de Haen, 32205, Germany
Xylol	Vogel 1.09843, Germany
Periodic Acid 0.5% sol	Merk 1.01646./2/, Germany
SCHIFF Reagent	Merk 1.01646./1/, Germany
Eosin 1%	Merk 1.15935.0100, Germany
Haematoxylin solution	Merk 1.05174, Germany
Neo-Mount	Vogel 1.09016, Germany
Entellan	Vogel 1.07961, Germany
Levamisol	Dako GmbH, Germany
LSAB-AP	Dako GmbH, k1018, Germany
LSAB-HRP	Dako GmbH, k1016, Germany
Acetyl- $\beta$ -Methacoline	Sigma GmbH, A2251, Germany
Methanol absolute	Roth GmbH, Germany
Milk powder	Roth GmbH, Germany
Percoll® / PAN-COLL mouse	PAN, P04-64500, Germany
Lympholyght M	Cedarlane, Nr.GL-5035, USA
CASY ton	Schärfe System, Reutlingen, Germany
CASY clean	Schärfe System, Reutlingen, Germany
Cytofix cell solution	BD Bioscience, Heidelberg, Germany
Cell fix solution	BD Bioscience, Heidelberg, Germany
BSA for Molecular Biology	Sigma GmbH, Germany
FACS Clean	BD Bioscience, Heidelberg, Germany

FACS Flow	BD Bioscience, 342003, Heidelberg, Germany
FACS Lyse	BD Bioscience, Heidelberg, Germany
<b>Buffers and solutions</b>	<b>Preparation</b>
OVA VI - Stock solution	20 mg OVA VI/1 ml PBS (Preserved in freezer at -20°C)
1% (w/v) OVA solution	1 mg OVA/100 µl PBS
25% (v/v) Al(OH) <sub>3</sub> solution	dilution Al(OH) <sub>3</sub> : PBS= 1:3
Sensitisation solution (i.p.)	dilution OVA 1%:Al(OH) <sub>3</sub> 25%= 1:1 Final conc. 10 µg/200 µl/application
Coating Solution (ELISA)	OVA VI Stock sol:PBS=1:1000 dilution Final concentration 20 µg/ml/well  (Always fresh make!)
Washing buffer (ELISA)	47.75 g PBS/5 ml Tween 20% solution/5 L d.Water (Always fresh make!)
Blocking solution (ELISA) 3% (w/v) BSA solution	15 g BSA/500 ml PBS (Preserved in freezer at -20°C)
Stop-reaction solution (ELISA) 2 M H <sub>2</sub> SO <sub>4</sub> solution	111 µl H <sub>2</sub> SO <sub>4</sub> 96% / 9889 µl d.Water
Lämmli-Puffer Stock solution (10x conc.) (WB)	10% SDS 1 g, 10% Saccharose (Sucrose) 1g  10% Glycerin 1ml in 0.1M Tris-HCl, pH 7.5 (1 ml HCl, 1 M/10 ml d.Water) add. 1-2 g Bromphenol Blue
Blocking buffer (WB)	5% Fish Gelatine or 5% Casein/ 1x TTBS (1x PBS/Tween 20)
TBS buffer Stock sol. (10x conc.) (WB)	0.2 M Tris-Base/1.5 M NaCl/1 L d.Water

TBS buffer solution (1x conc.) (WB)	100 ml TBS Stock/1 ml Tween20/1 L d.Water
3% (w/v) Milk solution (WB)	1.5 g Milk powder/50 ml PBS
Transfer buffer (Semidry buffer)(WB)	11.6 g Tris/Base/5.8 g Glycin/0.8 g SDS/400 ml EtOH abs/2 L d.Water
PBS solution (1xconc.) (WB)	9.55 g PBS powder/1 L d.Water
Dispersive gel-buffer B (WB)	1 g SDS/45.43 g Tris/Base – HCl/ 250 ml d.Water, until pH 8.8
Collecting gel-buffer C (WB)	1.5 g SDS/9 g Tris/Base – HCl/ 250 ml d.Water, until pH 6.8
Running buffer stock solution (10x conc) (WB)	20 g SDS/288 g Glycin/50 g Tris/Base /2 L d.Water
Running buffer (1x conc.) (WB)	Running buffer Stock solution (10x conc.):d.Water=1:10 dilution
Sample buffer Stock solution (10x conc.) (WB)	10 g SDS/30.3 g Tris/Base/144.1 g Glycerin/800 ml d.Water <b>(Aliquots preserved at -20°C)</b>
Sample buffer Stock solution (2x conc.) (WB)	10 ml Tris/Base of 1.5 M (pH=6.8)/ 6 ml SDS 20%/30 ml Glycerin/15 ml β-ME-EtOH/1.8 g Bromphenol blue/ 100 ml d.Water <b>(Aliquots preserved at -20°C)</b>
20% (w/v) SDS (WB)	10 g SDS/50 ml d.Water
10% (w/v) APS (WB)	10 g Amoniu Persulfat/100 ml d.Water <b>(Aliquots preserved at -20°C)</b>
Ponceau Stock solution (20x conc.) (WB)	4 g Ponceau/20 ml Acetic acid/ 100 ml d.Water
Ponceau solution (1x conc.) (WB)	Ponceau stock (20x conc.): d.Water =1:20 dilution
Anesthetic - Stock solution (5x conc.)	10 ml Ketanest (conc.50 mg/ml)/ 1.66 ml Rompun (conc.2%)

Equipment	Provider
Biofuge(Microcentrifuge)	Heraeus Instr.GmbH/Kobe,Marburg
Megafuge(Macrocentrifuge)	Heraeus Instr.GmbH/Kobe,Marburg
CO <sub>2</sub> Incubator cell culture	Heraeus Instr.GmbH, 22017449
FACSCalibur with Cell Quest Program	Becton Dickinson, San Jose, USA
Light-/Fluorescent Microscope	Olympus BX51, Japan
Light Microscope Olympus	Olympus, Japan, 250653
Microtommesser Feather Type S25	PFM AG, Germany 20750000
Ultrasonic Bath	Sonorex GmbH, 50013189
Water Bath	Köttermann GmbH, Germany
Tecan ELISA Reader	Biosource, Solingen, Germany
Head-out Body-Plethysmograph	ITEM Fraunhofer Institut for Toxicologie and Experimental Medicine, Hannover
CASY Cell Counter	Schärfe System, Reutlingen
Analyser System Modell TT	Schärfe System, Reutlingen
Spectrophotometer UV/Visile 3100pro	Biochrom GmbH, 81854
LightCycler Sistem Corporation	Roche Diagnostics, Indianapolis, USA

**Tab.3.8 Consumables, reagents, buffers, solutions and equipment used in the present doctoral study**



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#### **4.1 Intrauterine model: prenatal studies with experimental mouse model**

The study shows that the maternal immunity exercises an influence on development of the adaptive immune response in the offspring. This character finds possible already prenatally and operates in the early postnatal period. At the core of this character is the induction and instruction of a specific T-cell response in the child, taught by adaptive (Toll-like receptors) and innate (antibody) - regulatory pathways of the maternal immune system. An established model of pre-/postnatal (perinatal) allergen exposure is now described, involving molecular and cellular mechanisms with special emphasis on the prototypically Toll-like receptor bacteria and ligands investigated. These results demonstrated that maternal transfer of protection from development of allergic airway disease to offspring in our animal model to switch from T<sub>H</sub>2- into T<sub>H</sub>1-type immunity is exclusive antigen specific.

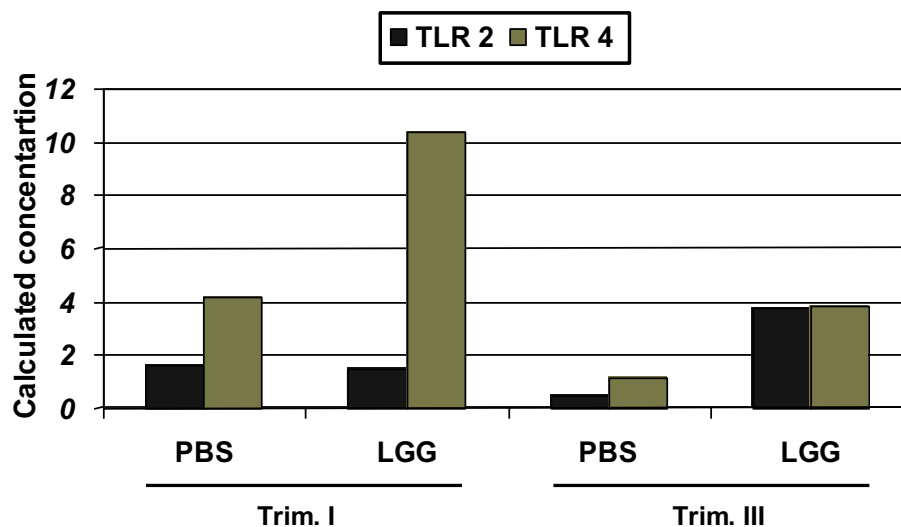
##### **4.1.1 Maternal immunosuppression effects biased by non-pathogenic bacterial exposure during pregnancy**

Reduced exposure to microbial allergens as a result of our hygienic daily life has been suggested as one of the possible causes. It has also been suggested that probiotics may provide safe alternative microbial stimulation needed for the developing immune system in infants. We established a non-pathogenic bacterial supplementation pregnant mouse protocol, in line to mimic the farming environment (aerosol and food allergens) exposure of pregnant woman and to initiate a potential protective molecular mechanism. We used Balb/c strain female mice, 6-8 weeks old, that received either intragastric (i.g.) *Lactobacillus rhamnosus* GG ( $10^8$  CFU per application, reconstituted in a total volume of 200  $\mu$ l PBS), intranasal (i.n.) Lipid A, the active component of LPS - *E.coli* (10  $\mu$ g per application, reconstituted in a total volume of 50  $\mu$ l of PBS) and intranasal (i.n.) *Acinetobacter lwoffii* ( $10^8$  CFU per application, reconstituted in a total volume of 50  $\mu$ l of PBS), from 10-8-6-4-2 days before conception when the maternal

supplementation starts and continued every second day until pregnancy term. Age-matched control animals received PBS.

#### 4.1.1.1 Maternal small intestine analysis: TLR2 and TLR4 mRNA expression

The intestine is the body's largest immune organ. Most of the antibody-producing cells reside in the intestine. Moreover, the intestinal microflora induce the maturation of the mucosal adaptive immune system, providing protection against potential pathogens, one suggesting mechanisms being TLR signaling pathway. Our animal model, prior prenatal - intragastrical supplementation of two different groups of female Balb/c strain mice, 6-8 weeks old, to *Lactobacillus rhamnosus* GG (LGG) and PBS (control group), on days 5 (Trimester I) and 18 (Trimester III) of pregnancy. Maternal small intestine is removed to perform the expression mRNA TLR and immunohistochemical analysis.

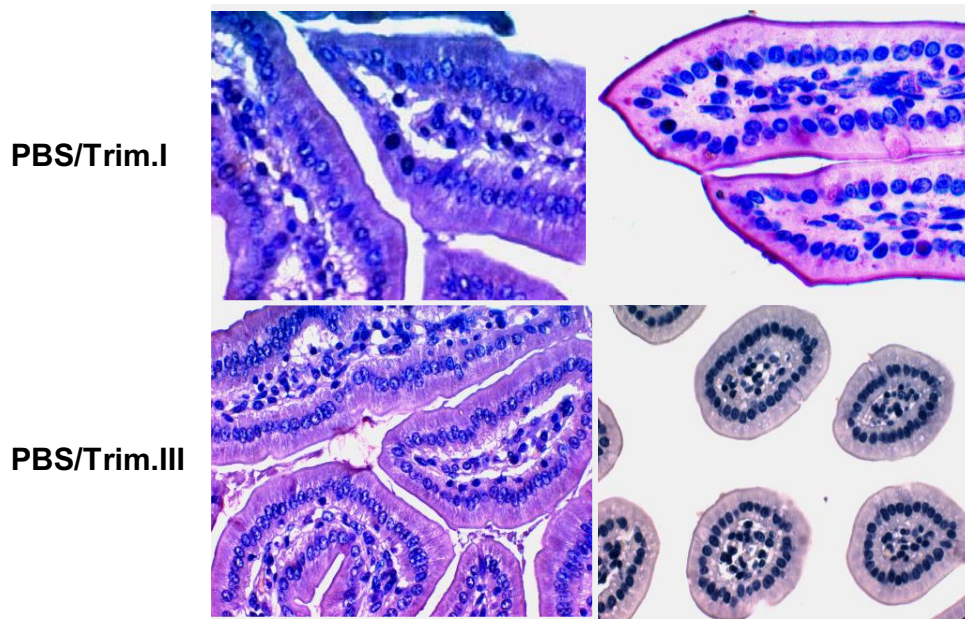


**Fig.4.1.1.1 Maternal small intestine mRNA TLR2 and TLR4 expression.** Maternal small intestine analysed at days 5 (Trim.I) and 18 (Trim.III) of pregnancy in the intervening time of non-pathogenic bacterial supplementation. TLR4 shows a trend in up-regulation by LGG in first trimester of pregnancy. TLR2 and TLR4 primers are provided by Prof. Kirsching Laboratory – TU München, Germany and Santa Cruz Company, USA (n=4 females/group)

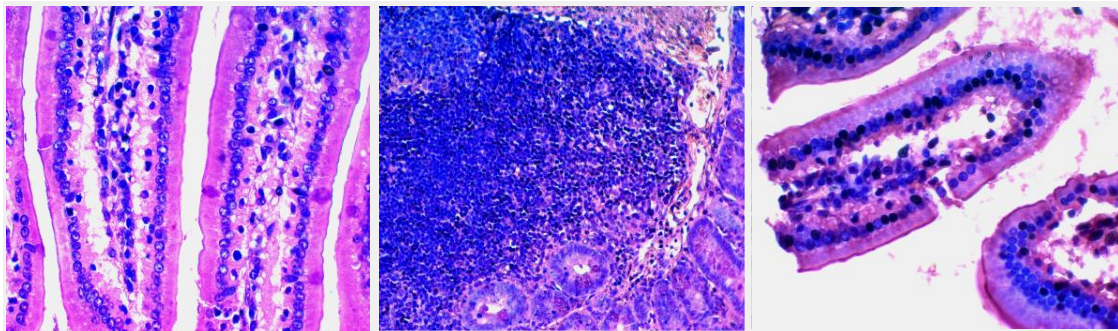
Small intestine mRNA TLR4 expression shown a high induction in the first trimester of pregnancy but not mRNA TLR2 in comparison with controls, while mRNA TLR2 and TLR4 expression in the third trimester of pregnancy is more equilibrate in both groups being influenced of other pregnancy molecular factors, like steroid hormones. Consequently, bacterial DNA is highly enriched in immunostimulatory CpG motifs that bias the immune response to antigens initiating a potential mechanism of maternal commensal gastrointestinal bacterial DNA to modulate a nonallergic T<sub>H</sub>1 immune response in infancy.

#### 4.1.1.2 Maternal small intestine analysis: TLR2 and TLR4 expression by Immunohistochemistry (IHC)

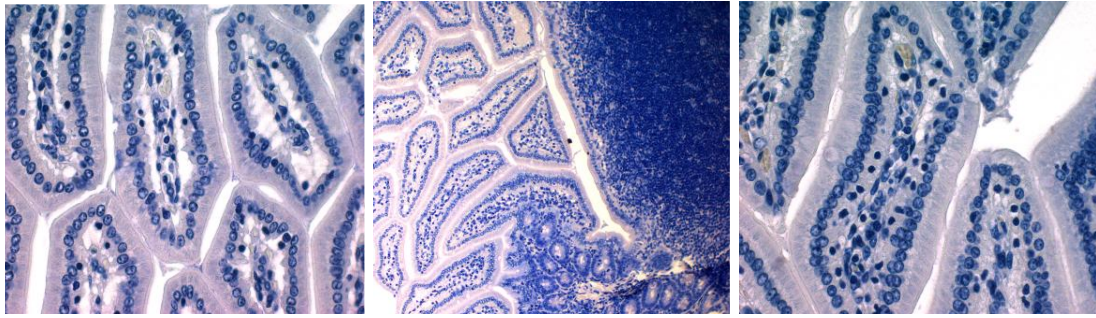
For immunohistochemistry analysis is necessary a preliminary preparation. Maternal gut is perfused with PBS buffer, pH 7.2 and fixed in situ with 4% (w/v) Formaldehyde solution. Entire tissue is paraffin-embedded or Tissue Teqpolymer cryopreserved. Later, gut tissue sections of 3 µm (paraffin-embedded gut tissue) and 10 µm thickness (frozen gut tissue), were obtain by fine tissue films cut using a Rotary Microtome and a Cryomicrotome, respectively. Indirect Immunohistochemistry staining for anti-mouse TLR2 in gut tissue was performed either with Horse Radish Peroxide (HRP) or Alkaline Phosphates (AP) systems as described in *Materials and Methods* chapter. Negative controls are performed on consecutive sections by replacing the primary antibody with a nonimmune mouse serum or PBS buffer. Tissue sections were examined by light-/fluorescent microscopy. Either in applied system such as HRP and/or AP staining, the TLR2 and TLR4 expression could not show differences between bacterial supplemented versus control groups. This can not be taking in consideration looking by the fluorescence staining. It can be observed that TLR2 is constantly expressed on maternal gut tissue surface, either in first and third trimester of gestation.



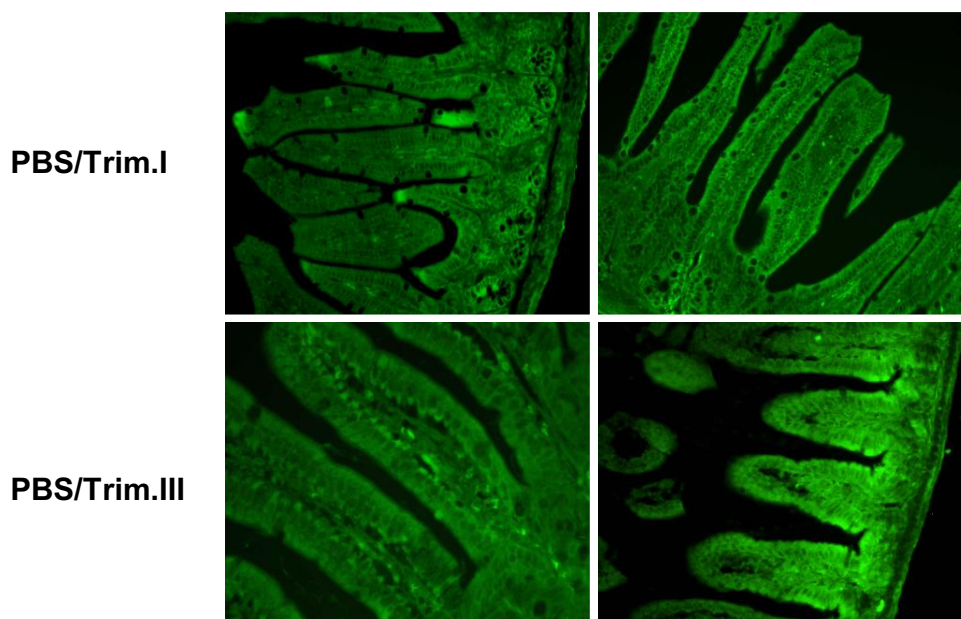
**Fig.4.1.1.2a Maternal small intestine immunohistochemistry analysis: TLR2 expression on duodenum of supplemented animals with PBS, in Trim.I/III of gestation** (first antibody, monoclonal rabbit anti-mouse TLR2 from Santa Cruz Co., USA and secondary antibody biotinylated goat - antirabbit Streptavidine peroxidase conjugated, M.O.M. Staining Set)



**Fig.4.1.1.2b Maternal small intestine immunohistochemistry analysis: TLR2 expression on jejunum, Peyer's patch and duodenum of supplemented animals with LGG, Trim.I of gestation** (first antibody, monoclonal rabbit anti-mouse TLR2 from Santa Cruz and secondary antibody, biotinylated goat – anti-rabbit / Streptavidine peroxidase conjugated, M.O.M. Staining Set)

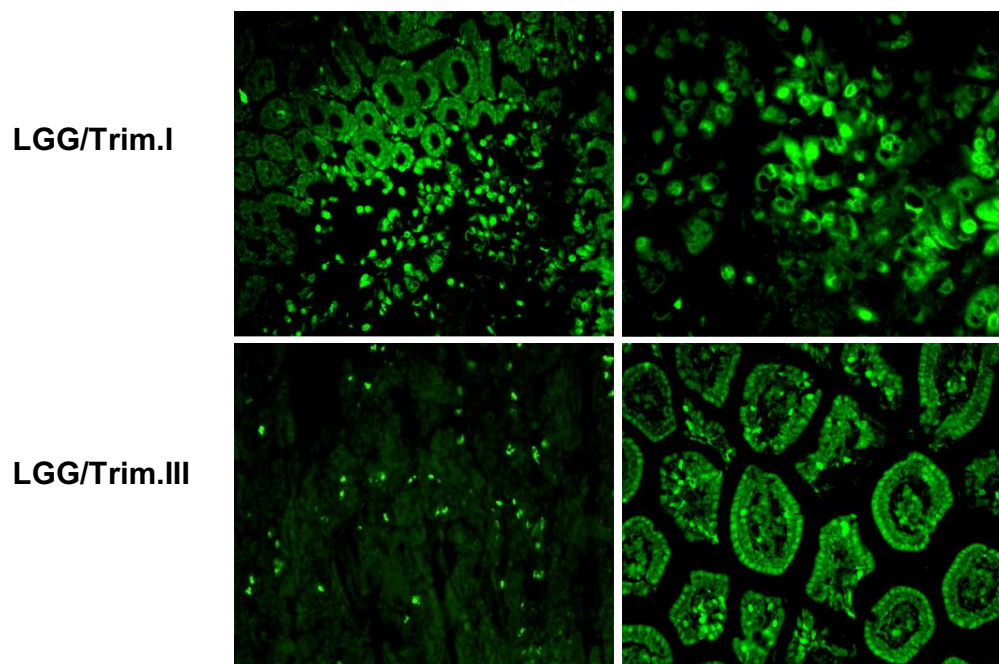


**Fig.4.1.1.2c Maternal small intestine immunohistochemistry analysis: TLR2 expression on jejunum, Peyer's patch and duodenum of supplemented animals with LGG, Trim.III of gestation** (first antibody, monoclonal rabbit anti-mouse TLR2 from Santa Cruz Co., USA and secondary antibody, biotinylated goat - antirabbit / Streptavidine peroxidase conjugated, M.O.M. Staining Set)



**Fig.4.1.1.2d Maternal small intestine immunofluorescence analysis: TLR2 expression by supplemented animals with PBS, Trim.I/III of gestation** (first antibody, monoclonal rabbit anti-mouse TLR2 from Santa Cruz Co., USA and secondary antibody, biotinylated goat antirabbit / Streptavidine – FITC conjugated, M.O.M. Staining Set)



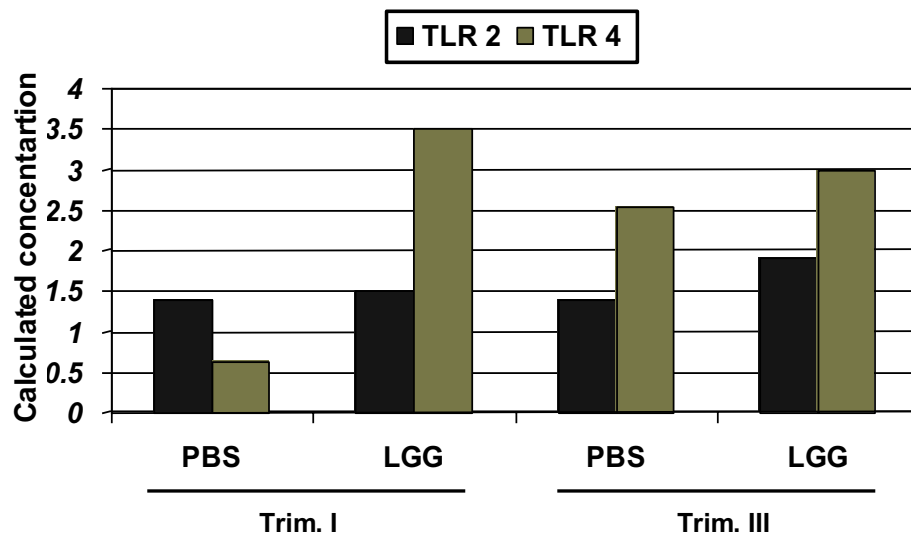


**Fig.4.1.1.2e Maternal small intestine immunofluorescence analysis: TLR2 expression by supplemented animals with LGG, Trim.I/III of gestation** (first antibody, monoclonal rabbit anti-mouse TLR2 from Santa Cruz Co., USA and secondary antibody, biotinylated goat - antirabbit / Streptavidine - FITC conjugated, M.O.M. Staining Set)

#### 4.1.1.3 Maternal spleen analysis: mRNA TLR2 and TLR4 expression

The spleen has a connective tissue capsule which spreads into the parenchyma forming splenic trabeculae. The parenchyma consists of white and red pulp. The white pulp is organized into periarteriolar lymphoid sheets (PALS, representing mainly T cells) and lymphoid follicles, which become prominent in response to stimulation. The periphery of the white pulp is formed by a less densely cellular marginal zone. The red pulp consists of reticular tissue and venous spaces (Hummel, 1975) and is the site of extramedullary hematopoietic activity, which normally occurs in the mouse spleen. Occasional accessory splenic tissue may occur in the pancreas. In our animal model, prior prenatal - intragastrical supplementation of two different groups of female Balb/c strain mice, 6-8 weeks old, to *Lactobacillus rhamnosus* GG (LGG) and PBS (control group), on days 5

(Trimester I) and 18 (Trimester III) of pregnancy. Maternal spleen is removed to perform the expression mRNA TLR and immunohistochemical analysis.

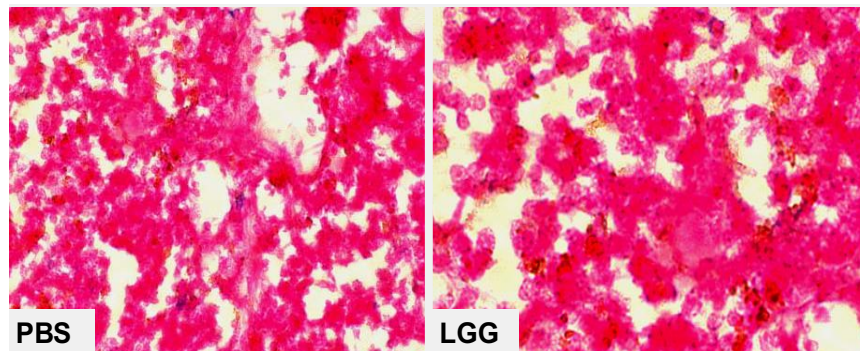


**Fig.4.1.1.3 Maternal spleen mRNA TLR2 and TLR4 expression.** mRNA TLR4 expression shows a trend in up-regulation by LGG only in first trimester of pregnancy (n=4 females/group)

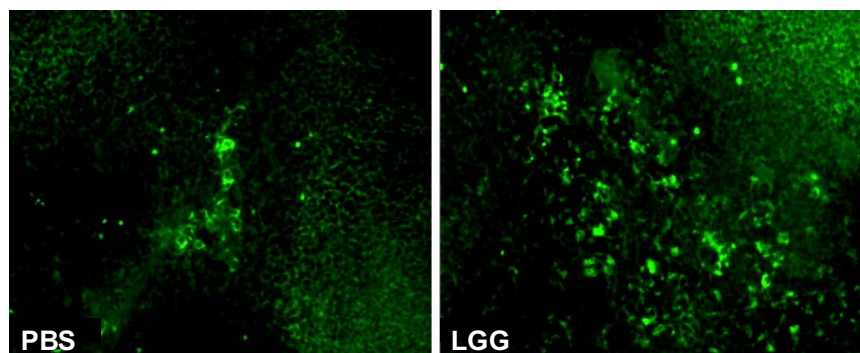
The spleen mRNA TLR 4 expression shown an induction in first trimester of pregnancy but not mRNA TLR2 in comparison with controls, while mRNA TLR2 and TLR4 expression in the third trimester of pregnancy is more equilibrate in both groups being influenced of other molecular factors, mostly the steroid hormones variability during the evolution of pregnancy.

#### 4.1.1.4 Maternal spleen analysis: TLR2 and TLR4 expression by Immunohistochemistry (IHC)

For immunohistochemistry analysis is necessary a preliminary preparation as describe for gut tissue. Indirect Immunohistochemistry staining is applied for anti-mouse TLR2 expression on maternal spleen tissue using either HPR or AP systems and examined by fluorescent microscopy. A significant difference between groups is not confirmed by mRNA TLR2 expression of the maternal spleen tissue, at term (Trim.III)



**Fig.4.1.1.4a Immunohistochemistry (IHC) of maternal spleen anti-mouse TLR2 after LGG prenatal supplementation - gestation at term** (first antibody, monoclonal rabbit anti-mouse TLR2 provided by Prof. Kirsching Laboratory – TU München, Germany and Santa Cruz Co., USA; secondary antibody, biotinylated goat – anti-rabbit/ Streptavidine – HPR conjugated, M.O.M. Staining Set).



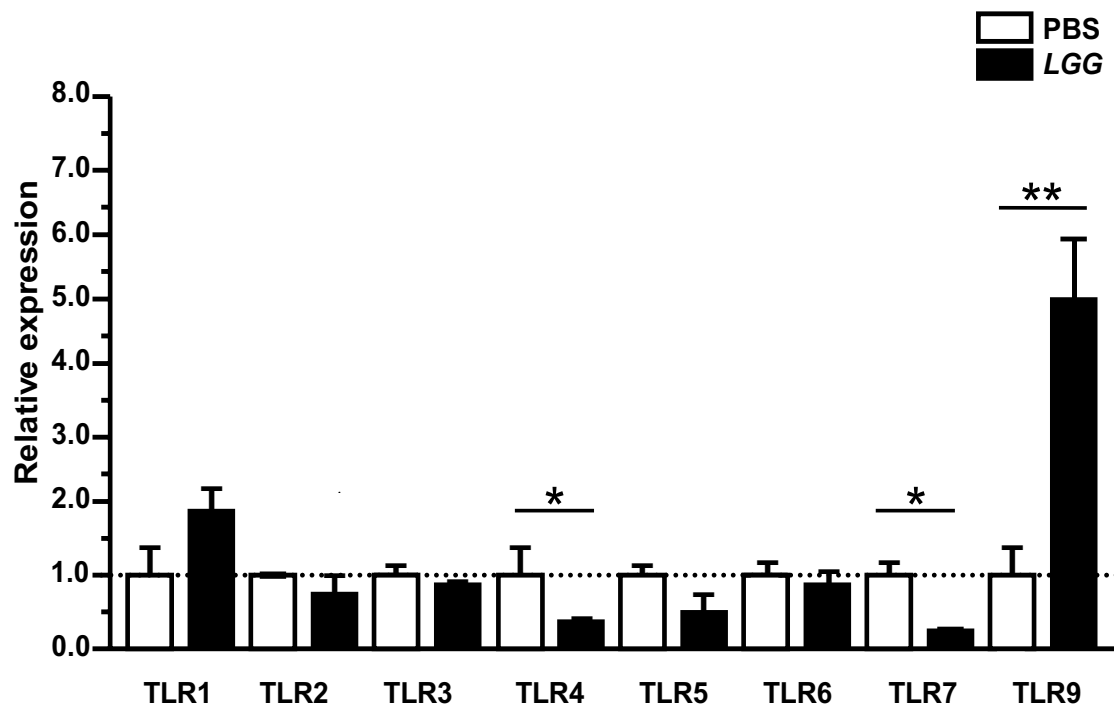
**Fig.4.1.1.4b Immunofluorescence (IF) staining of maternal spleen anti-mouse TLR2 after LGG prenatal supplementation – gestation at term** (first antibody, monoclonal rabbit anti-mouse TLR2 provided by Prof. Kirsching Laboratory – TU München, Germany and Santa Cruz Co., USA; secondary antibody, biotinylated goat – anti-rabbit/ Streptavidine – FITC conjugated, M.O.M. Staining Set).

Consequently, the immunohistochemical analysis of maternal spleen tissue, anti-mouse TLR2 expression, respectively does not reveal any significant variation between supplemented *versus* control groups.

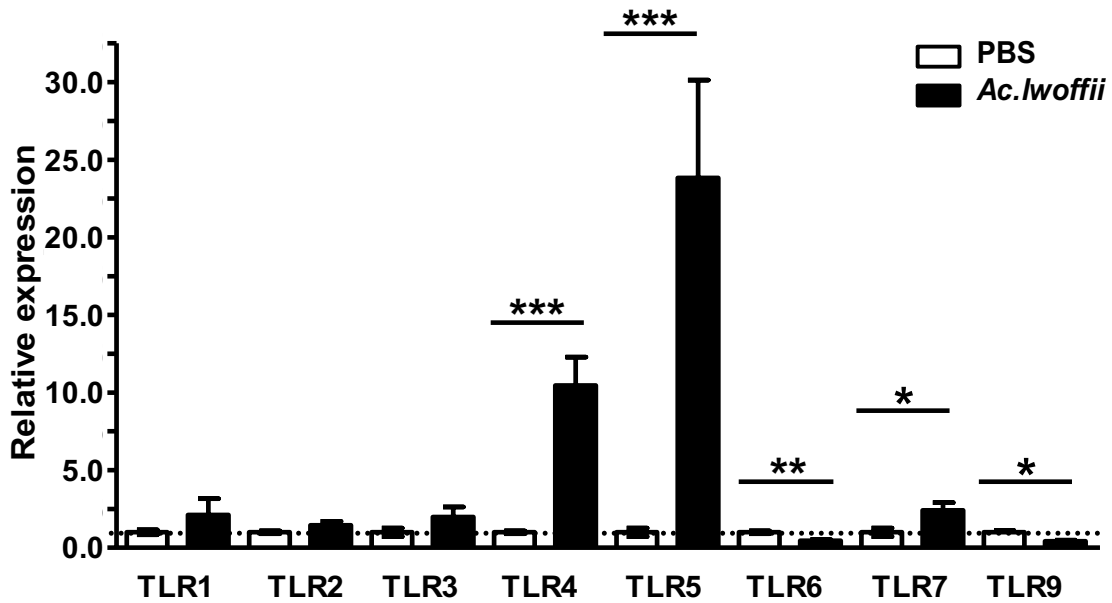


#### 4.1.1.5 Term Placenta Tissue Analysis: mRNA TLR expression

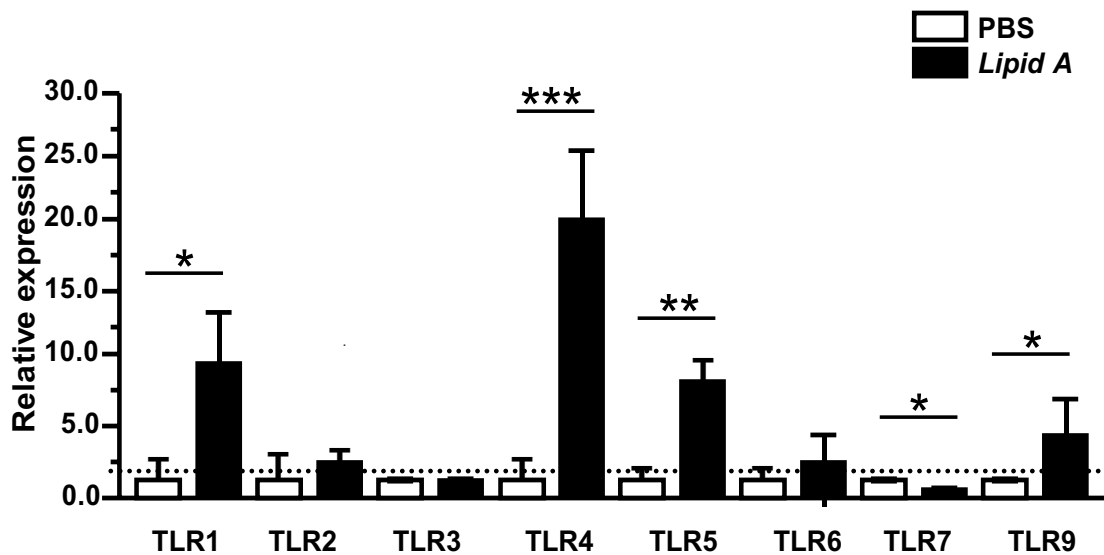
RNA was isolated from maternal organs, such as spleen and placenta via the TriFast method (Peglab, Erlangen, Germany). Previously, 50-100 mg of organs are perfused with PBS, freeze in liquid nitrogen avoiding the RNA degradation at room temperature and homogenized in TriFast lysis buffer, after disruption tissues being done mechanically using a mortar and pestle. The Reverse Transcription and qRT-PCR are performed in order to obtain expression of mRNA - TLR, cytokines and chemokine. Variation in placental mRNA TLR expression is seen in supplemented Balb/c mice when compared with controls. Steroid hormones, host cell - intracellular TLR non-/dependent molecular mediators signalling pathway and bacterial CpG could be involved in the variation of TLR signalling (Fig.4.1.1.5a, b, c).



**Fig.4.1.1.5a Variation in placental mRNA-TLR expression in LGG supplemented group versus control.** TLR4 and TLR7 are suppressed, while TLR9 expression is high induced (\* $P < 0.05$  and \*\* $P < 0.01$  vs. control; shown are mean  $\pm$  SEM;  $n = 4$  female/study group)



**Fig.4.1.1.5b Variation in placental mRNA-TLR expression in *Ac. Iwoffii* supplemented group versus control.** TLR6 and TLR9 are suppressed, while TLR4, TR5 and TLR7 expression is very high induced. (\*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 versus control; shown are mean  $\pm$  SEM; n= 10 females/study group)



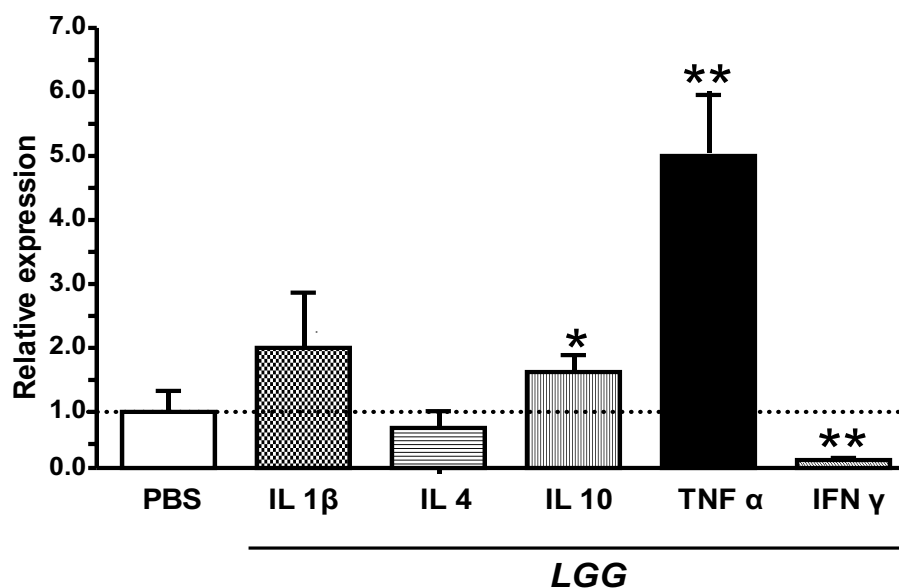
**Fig.4.1.1.5c Variation in placental mRNA-TLR expression in Lipid A supplemented group versus control.** TLR7 is suppressed, while TLR1, TLR4, TLR5 and TLR9 expression is high induced (\*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 versus control; shown are mean  $\pm$  SEM; n= 8 females/study group)

In LGG supplemented group mRNA TLR4 and TLR7 are suppressed, while TLR9 expression is high induced. Also, *Ac.lwoffii* supplemented group shown that TLR6 and TLR9 are suppressed, whereas TLR4, TLR5 and TLR7 expression is high induced. Variation in placental TLR mRNA expression in LA supplemented group in comparison with control we also observed. TLR7 expression is down-regulated, at the same time as TLR1, TLR4, TLR5 and TLR9 signals are significantly induced. Consequently, trends in LGG and LA supplemented mice embrace increasing TLR1 and TLR9, and decreasing of TLR7 expression, while in *Ac.lwoffii* group TLR7 expression is up-regulated and at the same time the TLR6, respective TLR9 signals are suppressed. Differences between supplemented groups include a significant increase only in TLR4 and TLR5 expression in LA and *Ac.lwoffii* supplemented mice, opposing to LGG supplemented group which reveal a significant suppression of mRNA TLR4 expression.

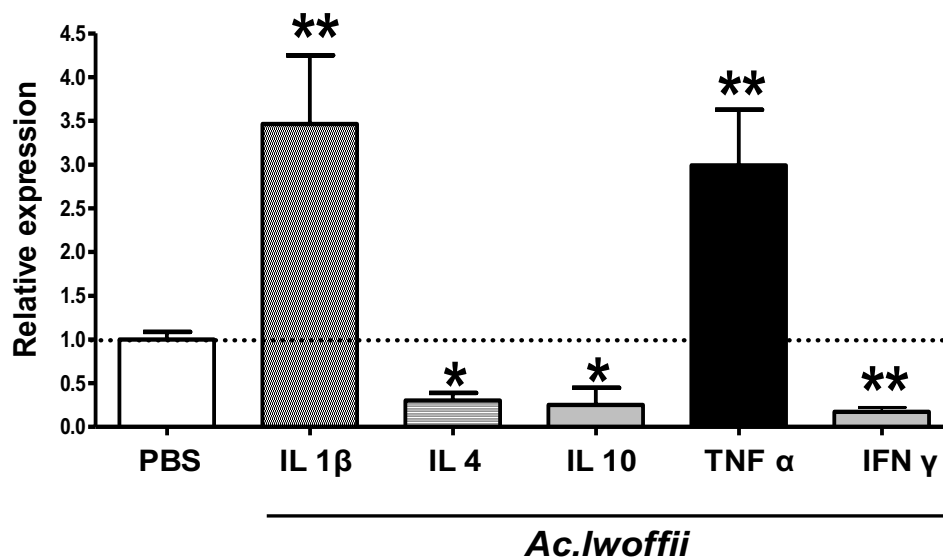
#### **4.1.1.6 Term placenta tissue analysis: mRNA $T_H1/T_H2$ cytokine and chemokine expression**

Furthermore, differences in mRNA  $T_H1/T_H2$  cytokine expression were also considering. There is a balance between  $T_H1/T_H2$  cytokine which can successful maintain pregnancy. The cytokine expression could be promoted either by trophoblasts and maternal-foetal interface lymphocytes. The analysis is performed as described in expression of mRNA TLR. Also, other studies (Szekeres-Bartho, 1989 and 1990) demonstrated that, steroid hormones have a consistent contribution of placenta's tissue cytokine expression from implantation to term of pregnancy. Here, the LA, the active component of LPS, is leading a biochemical instruction during the systemically absorption, activating the lipid metabolism, whereas the supplementation with entire bacteria organisms (LGG and *Ac.lwoffii*) revealed us opposing results. Accordingly, as regulators of lipid and lipoprotein metabolism, glucose homeostasis and cellular differentiation, in 1990 were identified Peroxisome Proliferator-Activated Receptors (PPARs) and

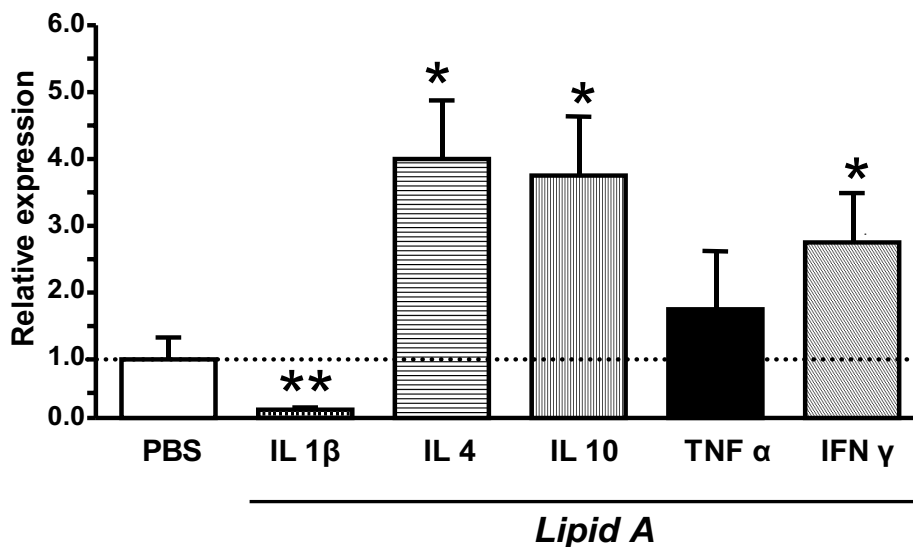
indicate their implication in cancer development as well as in the control of the inflammatory response and inflammation-related disorders. All PPARs heterodimerize with the *retinoid X receptor* (RXR) and bind to specific regions on the DNA of target genes. These DNA sequences are termed Peroxisome Proliferator Hormone Response Elements (PPREs). The DNA consensus sequence is AGGTCA $\mathbf{X}$ AGGTCA, with  $\mathbf{X}$  being a random nucleotide. In general, this sequence occurs in the promotor region of a gene, when the PPAR binds its ligand, transcription of target genes is increased or decreased, depending on the gene. But the function of PPARs is modified by the precise shape of their ligand-binding domain, induced by ligand binding and by a number of coactivator and corepressor proteins, the presence of which can stimulate or inhibit receptor function, respectively.



**Fig.4.1.1.6a Evaluation of placental mRNA  $T_H1/T_H2$  cytokine expression in LGG supplemented group versus control.** The variation between LGG and PBS groups is observed in high expression of TNF $\alpha$  and IL-10, while IFN $\gamma$  is strongly suppressed (\* $P < 0.05$  and \*\* $P < 0.01$  vs. ctrl; shown are mean  $\pm$  SEM;  $n = 4$  females/study group)

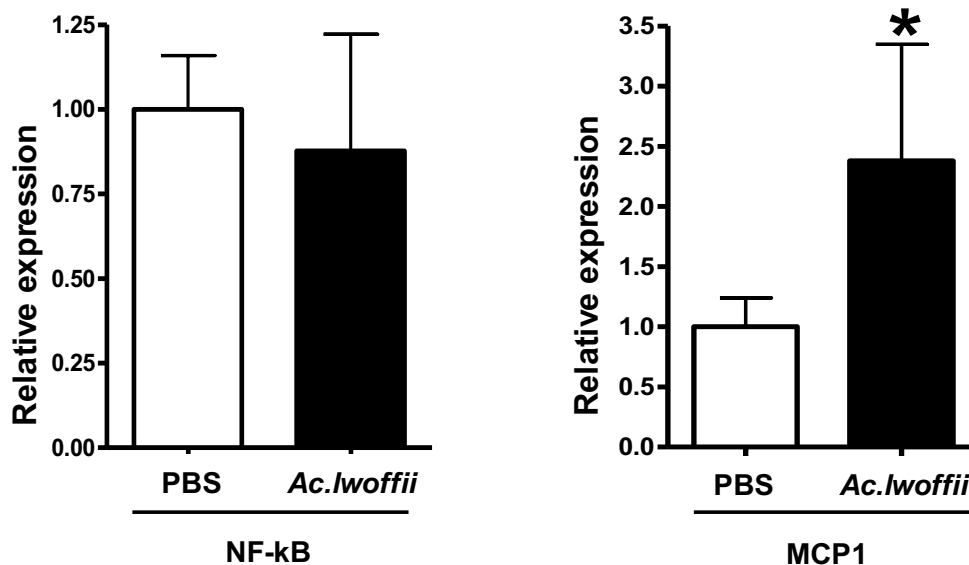


**Fig.4.1.1.6b Evaluation of placental mRNA  $T_H1/T_H2$  cytokine expression in *Ac. Iwoffii* supplemented group versus control.** The variation between *A. Iwoffii* and PBS groups is observed in high expression of TNF $\alpha$  and IL-1 $\beta$ , while IL-4, IL-10 and IFN $\gamma$  are strongly suppressed. (\* $P < 0.05$  and \*\* $P < 0.01$  vs. ctrl; shown are mean  $\pm$  SEM;  $n=10$  females/study group).

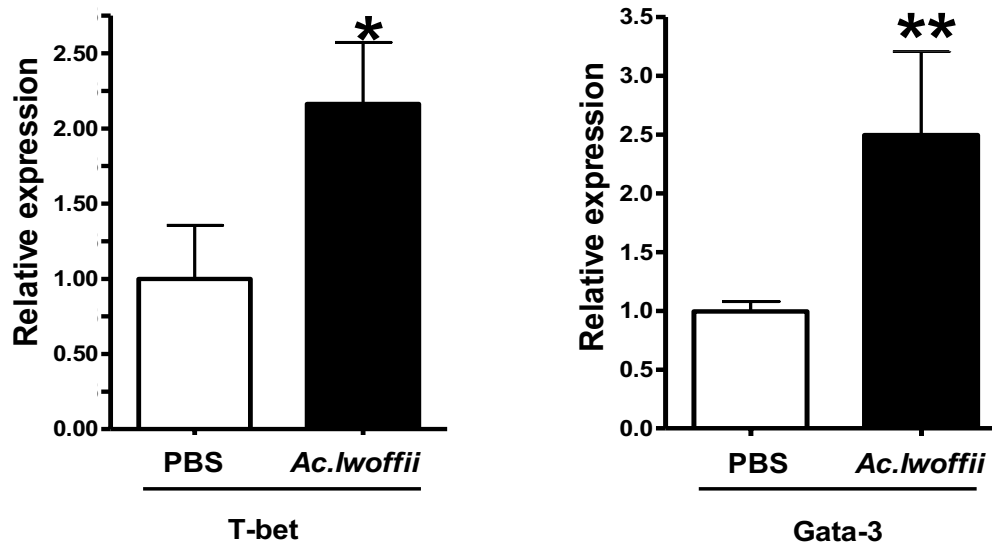


**Fig.4.1.1.6c Evaluation of placental mRNA  $T_H1/T_H2$  cytokine expression in Lipid A supplemented group vs control.** The variation between Lipid A and PBS groups is observed in high expression of IL-4, IL-10 and IFN $\gamma$ , while IL-1 $\beta$  is strongly suppressed (\* $P < 0.05$  and \*\* $P < 0.01$  vs. ctrl; shown are mean  $\pm$  SEM;  $n=8$  females/study group)

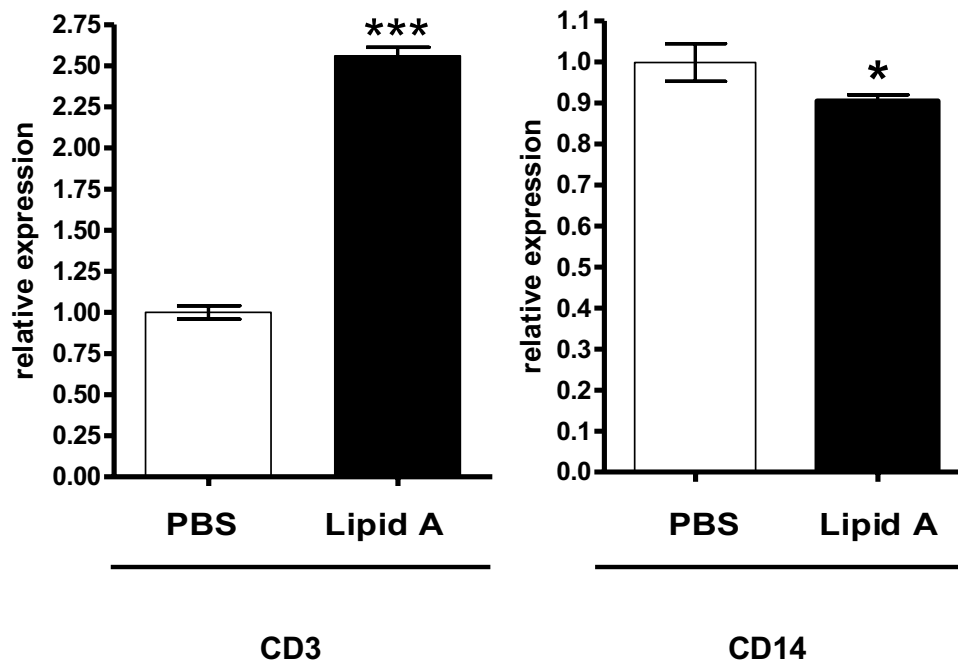
Trends in supplemented mice include an increase in placental T<sub>H</sub>2 cytokines such as IL-4 and IL-10 in LGG, respective LA supplemented groups, at the same time as *Ac. Iwoffii* group shown a significant decrease of these cytokines. Furthermore, the LGG and *Ac. Iwoffii* groups demonstrated an increase in pro-inflammatory cytokines like IL-1 $\beta$ , whereas a down-regulation of this cytokine in LA supplemented animals is observed. Also, significant differences between all supplemented groups include an inverse relationship between IFN $\gamma$  and TNF $\alpha$ . Certain adaptor molecules and TLR signalling mechanisms mediators, transcription factors and chemokine (NF- $\kappa$ B, MCP-1, T-bet, Gata-3, FoxP3, CD3, CD14, CD11) which play an immunomodulatory role in inflammatory response and revealed an induction into T<sub>H</sub>1 response at placental mRNA level, were evaluated in groups supplemented either with LA and *Ac. Iwoffii* F78, in comparison with controls.



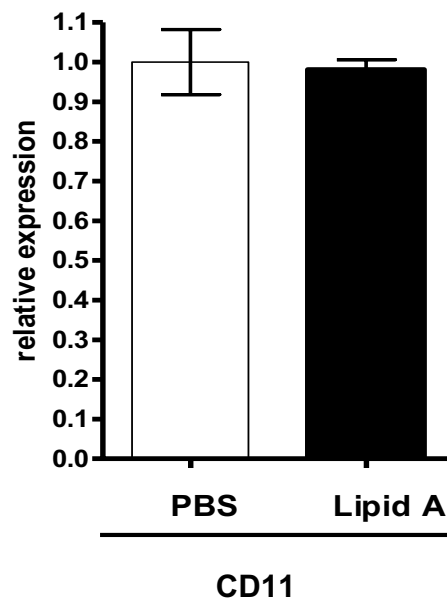
**Fig.4.1.1.6d Variation of mouse placental transcription factors and chemokine NF- $\kappa$ B, MCP-1 (equivalent of IL-8 in human) at mRNA level in *Ac. Iwoffii* supplemented group in comparison with control.** It is also well known, that the complement system's main function is to annihilate foreign pathogens. The pathway is tightly regulated by some coactivator proteins (membrane cofactor protein1, MCP-1) at the key steps of initiation, amplification and membrane attack to avoid the tissue injury (\*P< 0.05 and \*\*P< 0.01 vs. ctrl; shown are mean  $\pm$  SEM; n=10 females/study group).



**Fig.4.1.1.6e** Variation of placental transcription factors T-bet, Gata-3 mRNA expression in *Ac. Iwoffii* supplemented group in comparison with controls. FoxP3 mRNA do not show a significant difference between supplemented and control groups. (\* $P < 0.05$  and \*\* $P < 0.01$  vs. ctrl; shown are mean  $\pm$  SEM;  $n = 10$  females/study group).



**Fig.4.1.1.6f** Variation in placental mRNA CD3 and CD14 expression in Lipid A supplemented group in comparison with control. CD3 mRNA expression is strong up-regulated, while the CD14 mRNA shown a trend in downregulation and CD11 mRNA expression do not reveal any differences between groups. (\* $P < 0.05$  and \*\* $P < 0.01$  vs. ctrl; shown is mean  $\pm$  SEM;  $n = 8$  females/study group)

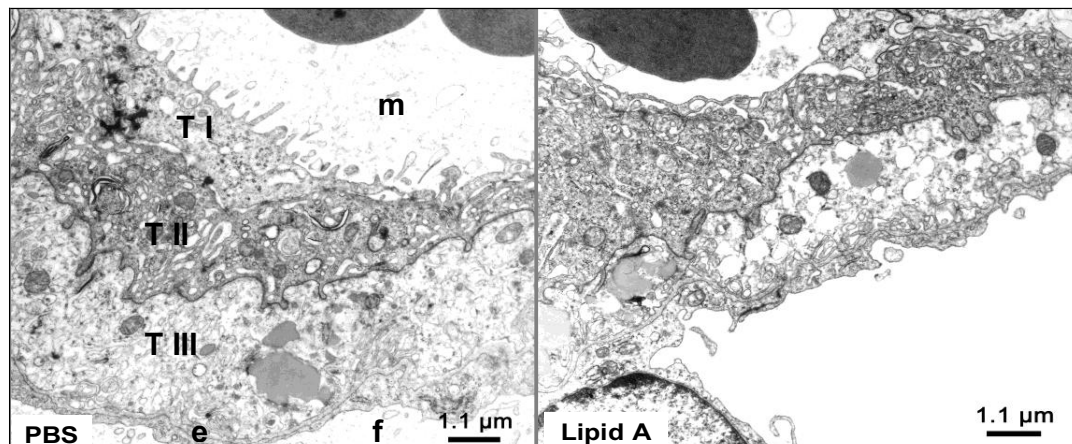


**Fig.4.1.1.6g Variation in placental mRNA CD11 expression in Lipid A supplemented group in comparison with control.** CD3 mRNA expression is strong up-regulated, while the CD14 mRNA shown a trend in downregulation and CD11 mRNA expression do not reveal any differences between groups. (\* $P < 0.05$  and \*\* $P < 0.01$  vs. ctrl; shown is mean  $\pm$  SEM;  $n=8$  females/study group)

#### 4.1.1.7 Microscopical examination of mouse placenta

The recent insight that the intrauterine environment has a profound effect on predisposition to chronic illness later in life (Barker, 2004) has led to a high interest in placental development and function. Furthermore, because of the ease of genetic manipulation, the mouse is increasingly being used as an experimental model system to investigate placental development. In our prenatal study, the immunology of placenta was examined in detail, to acquire an underlying potential maternal immune-protective molecular mechanism to her progenies against allergies. Significant morphological differences exist between the human and the mouse placentas, but at the ultra-structural level comparisons are limited because little information was available up to know for the mouse (Enders, 1965. Kirby & Brandbury, 1965. Björkman, 1970. Martinek, 1971). In our study, in collaboration with Prof. Dr. H.Fehrenbach we performed a transmission electron microscopy in an ultra-structural description of murine placenta, either in control and supplemented animals. We followed the method used in Prof. Fehrenbach Laboratory as described in *Materials and Methods* chapter.

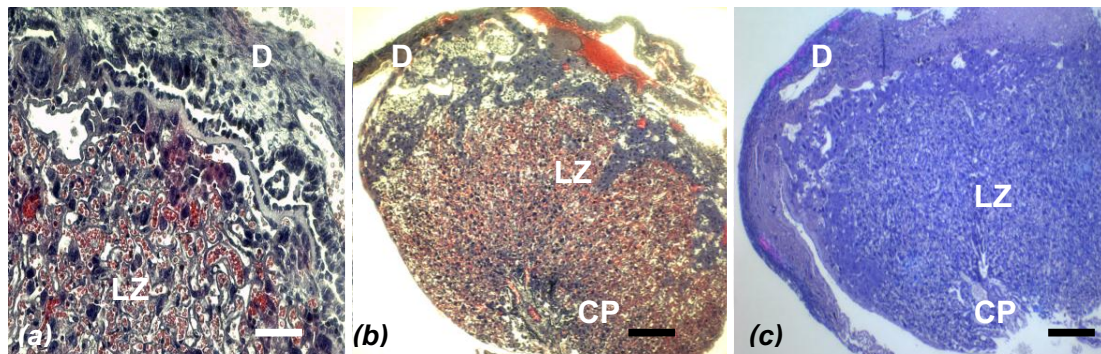




**Fig.4.1.1.7a Placenta electro-micrographs: Transmission electron micrograph of the placental barrier shown in Lipid A group versus control.** Pictures illustrate the interaction between trophoblasts layers and blood spaces within the labyrinth. Note the three trophoblast layers (I, II, and III) and fetal endothelial cell (e) separating the maternal (m) and fetal (f) blood spaces. Analysis performed with assistance of Prof.Dr. Heinz Fehrenbach, Clinical Research Group "Chronic Airway Diseases", Clinic of Internal Medicine and Respiratory Medicine, Philipps University of Marburg / Lahn, Germany.

The term placenta contains cells of both fetal and maternal origin, subdivided into three zones. Closest to the fetus is the labyrinthine zone (LZ), which consists of a complex interconnecting system of maternal blood spaces separated by trabecular cords of fetal tissue containing capillaries. Three layers of fetally derived trophoblast separate the fetal capillaries from the maternal blood, and hence the murine placenta is classified as haemotrichorial. The outer layer of trophoblasts bathed directly by maternal blood (trophoblast layer I, TI) is cellular mononucleated, the cytotrophoblast (CT), whereas both the inner two layers (trophoblasts layers II and III, TII and TIII) are multinucleated – syncytiotrophoblast (ST) (Mossman, 1987). The LZ is playing a main role in nutrients, gas and waste are exchanged between mother and fetus, and as gestation progresses this compartment occupies the majority of the placental area (Coan et al. 2004). However, the exact contribution each component of the trichorial membrane makes towards facilitation or impedance of exchange has yet to be revealed. Separating the labyrinthine zone from the maternal uterine

tissue is the basal / junctional zone (JZ). This zone is a cellular compartment consisting of at least two distinct cell types after embryonic day E12.5: spongiotrophoblast (SPT) and glycogen cells (GCs). Both these cells express the genetic marker *Tpbpa* and the spongiotrophoblast marker *Ascl2* (Cross, 2005). The function of this zone is yet to be fully understood (Georgiades, 2002), but it is essential for the fetal survival as demonstrated by the *Ascl2*<sup>-/-</sup> mouse that lacks this zone and is lethal around E10.5 (Guillemot, 1994). Bordering the JZ and the maternal decidua is a unicellular discontinuous layer of trophoblasts giant cells (GCs), (Pijnenborg, 1981). They are distinct from cells of the JZ in expressing the giant cell-specific marker *Hand1* (Hemberger & Cross, 2001). These large mononuclear, polyploidy (Zybina & Zybina, 1996) trophoblasts produce a variety of hormones and chemokines, (Malassiné, 2003), which may alter maternal physiology and immunology in order to accommodate the fetal allograft. Furthermore, the structure of the murine placenta at the gross morphological and light microscopically levels has been reviewed by other groups, too (Georgiades, 2002 and Malassiné, 2003), as have the molecular mechanisms supporting cell lineage, proliferation and differentiation (Rossant & Cross, 2001; Downs, 2002). In our experimental protocol, after animal sacrifice the placentas were cut transversally and fixed in situ with Formaldehyde 4% (wt/vol) sol. Both pieces of placenta tissue was Paraffin-embedded or Tissue Teq polymer cryopreserved and 3 µm, respective 10 µm sections, previously cut by using a Rotary Microtome and Cryomicrotome were stained with Haematoxylin and Eosin (H&E) for tissues characterization and cell profiles of inflammatory cells state via High-power light microscopy (HPLM). Indirect Immunohistochemistry staining such as Horse-Radish Peroxidase (HPR) or Alkaline-Phosphate (AP) systems for anti-mouseTLR2 and anti-mouseTLR4 on lymphocytes and/or trophoblasts is used. Samples are examined by Fluorescent microscopy (FM), (Fig.4.1.1.7b, c, d, e).

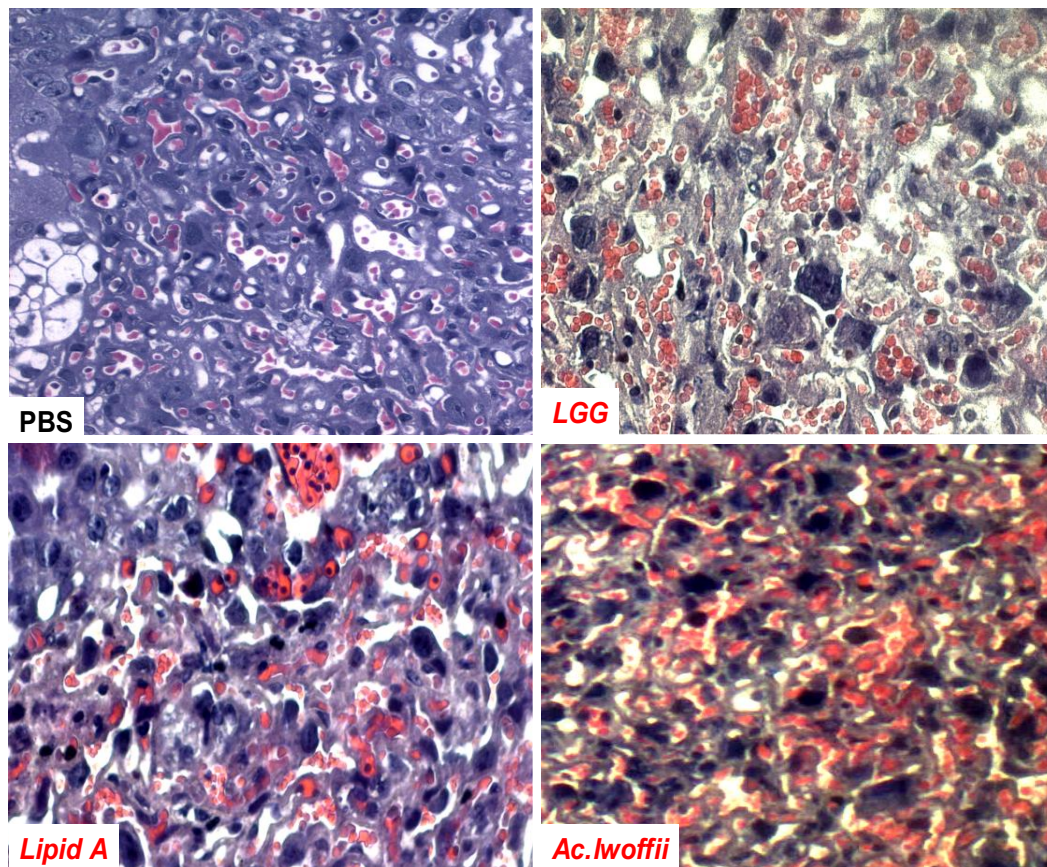


**Fig.4.1.1.7b Placenta histology – sagittal sections - from LGG (a), Ac.Iwoffii (b), Lipid A (c) supplemented groups.** Placenta tissues illustrate the same architecture: unmodified structure and a normal alignment of the specific trophoblasts layers. Magnification of 10x and 4x by light microscopy.

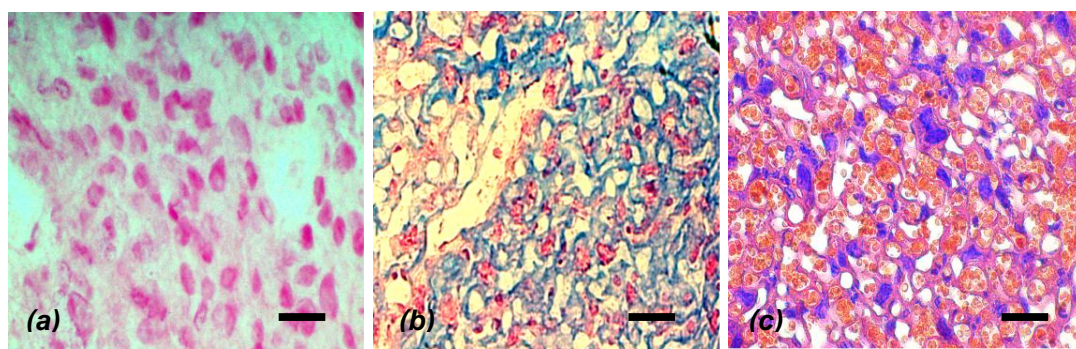
It is obviously that closest to the fetus (umbilical blood cord) is the chorionic plate (CP) and the labyrinthine zone (LZ), which consists of a complex interconnecting system of maternal blood spaces separated by trabecular cords of fetal tissue, containing capillaries. Three layers of fetally derived trophoblast (one layer – cytotrophoblast, respective two layers of derived syncytiotrophoblasts) separate the fetal capillaries from the maternal blood, and hence the murine placenta is classified as haemotrichorial.

On the opposite side, the decidualised trophoblasts (D) are naturally developed, playing the most important role in implantation, maternal immunosuppression, maintaining the  $T_H1/T_H2$  balance and at term of pregnancy, being first cells signaling the labor process or baby delivery.

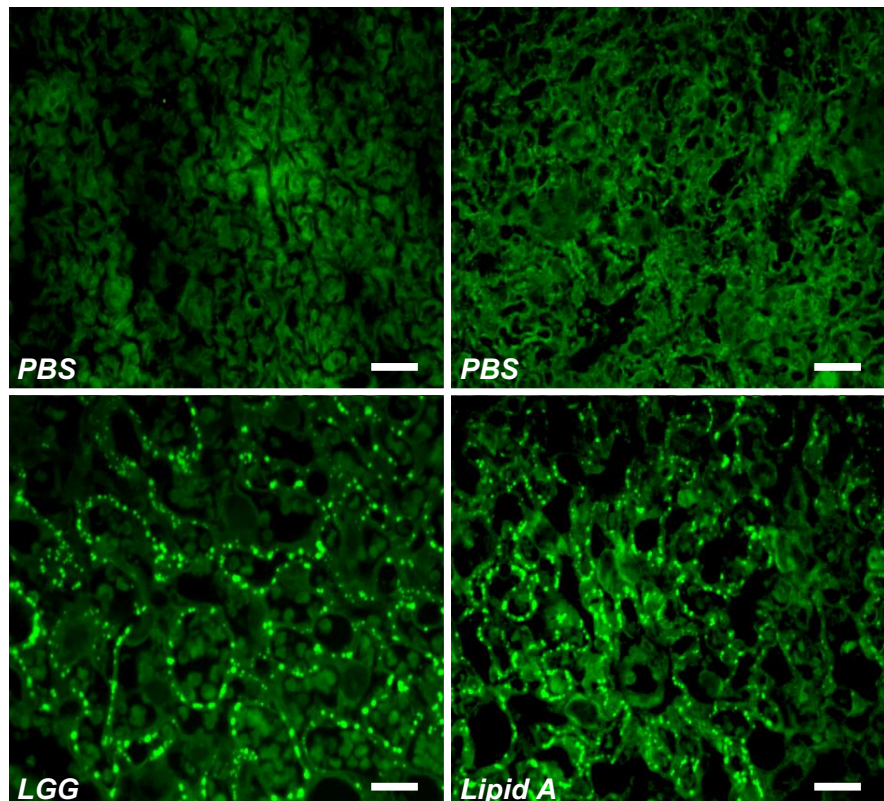




**Fig.4.1.1.7c Placenta histology – the labyrinth (H&E staining) of PBS, LGG, *Ac. Iwoffii*, Lipid A supplemented groups.** Placenta tissues show the same architecture: unmodified structure and a normal alignment of the specific trophoblasts layers. Magnification of 20x by light microscopy.



**Fig.4.1.1.7d Immunohistochemistry: Placental anti-mouse TLR 2 – FITC, M.O.M kit – AP and HRP staining systems.** Tissues of PBS (a), LGG Gram<sup>+</sup> bacterium (b) and Lipid A/LPS-*E.coli* Gram<sup>-</sup> bacterium (c) supplemented groups could not be specific stained for TLR2. Light microscopy is used with magnification of 20x.



**Fig.4.1.1.7e Immunofluorescence: Placental anti-mouse TLR 2 – FITC, M.O.M system.**

Mouse placenta electronmicrographs illustrate the placental barrier architecture in very well-designed way. Both tissues of control and treated mother mice reveal an identical structure, consequently the supplementation did not influence placenta histomorphology, but very much its physiology as is demonstrated at the molecular experimental level. Nevertheless, the expression of placental TLR2 at the protein level by IHC could not be properly detected since a specific antigen-retrieval and an enzymatic inhibitor for peroxidase or alkaline phosphatase applied on placental tissue are not commercial available. Fluorescence microscopy is used with magnification of 20x.

Thus, the whole knowledge obtained by ultra-/gross microscopy of the mouse placenta - the trophoblast subclasses (placental barrier), respective the detailed description of the placental layers/trophoblast classes (fetal and maternal cell lineages, feto-maternal interface), allowed us to understand the cellular and molecular aspects, potential pathways involved into maternal protective mechanism.

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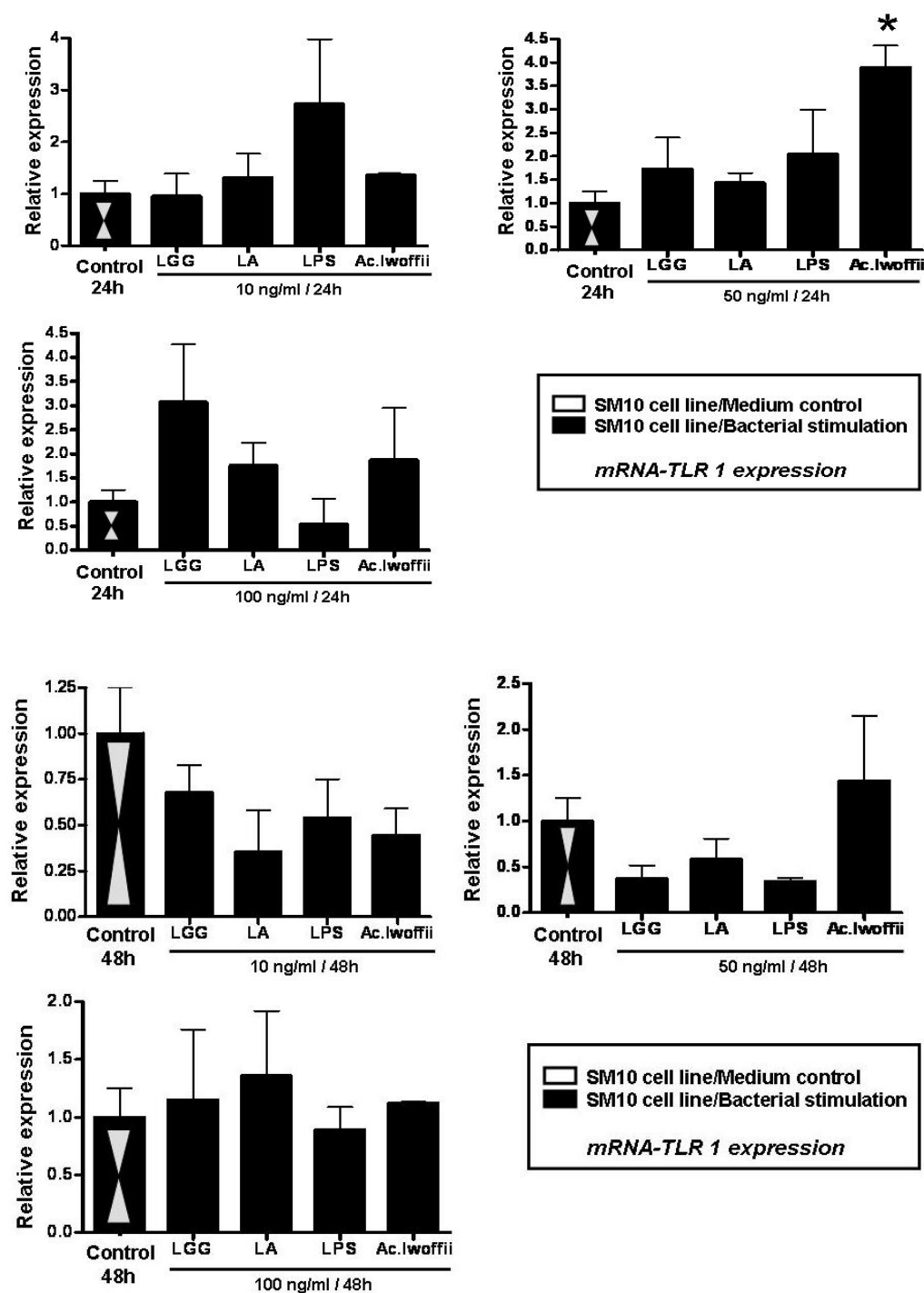
#### 4.1.2 Placenta's fetal side: trophoblast stem cells analysis

Trophoblast Stem Cells (TSCs), SM10 cell line are still present in term placenta labyrinth, under the chorionic plate and could be maintained undifferentiated but commitment state in cell culture. The cells were passage several weeks until the cell number was adequate for stimulation and then, four different allergens such as *Lactobacillus rhamnosus* GG (LGG), Lipid A (LA-LPS/*E.coli*), Lipopolysaccharide (LPS/*E.coli*) and *Acinetobacter lwoffii* F78 (*Ac.lwoffii*) with concentrations of 10, 50, 100 ng/1ml cell suspension, were applied for 24 and 48h incubation time. The cell-free supernatants were harvested and levels of IL-4, IL-10, IL-1 $\beta$ , TNF $\alpha$ , IFN $\gamma$  were measured using Enzyme-linked immunosorbent assay (ELISA).

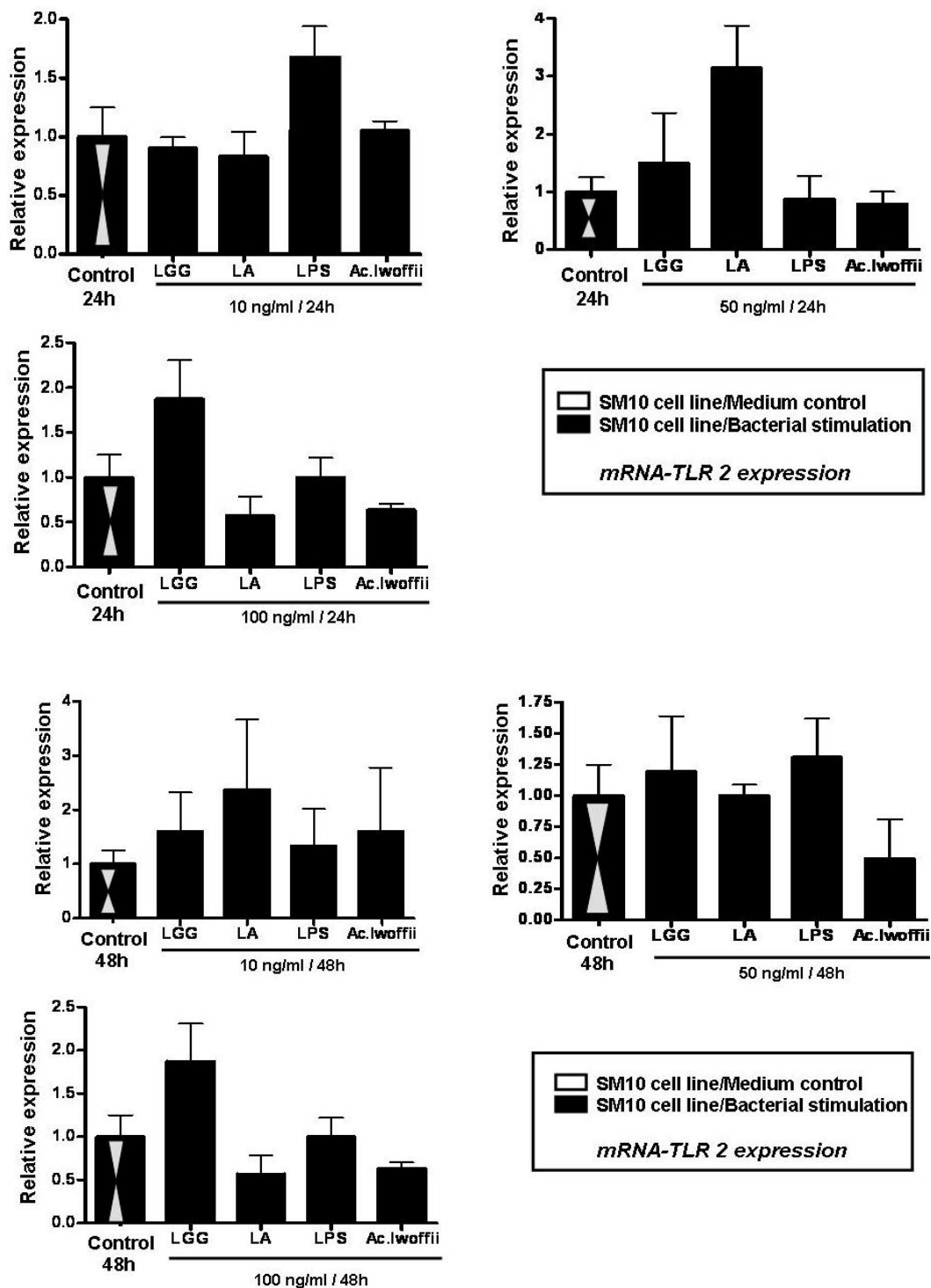
##### 4.1.2.1 Trophoblast stem cells analysis: mRNA TLR, cytokine and chemokine expression after bacterial restimulation in culture

Placental - Trophoblast Stem Cells (TSCs), SM10 cell line is passage several weeks until the cell number was sufficient for stimulation. After trypsination followed by washing steps, the cells were counted by CASY TT Cell Counter System, and  $1 \times 10^6$  cells/ml/well cultured in 24-well flat-bottom plates at 37°C in a cell incubator, under 5% CO<sub>2</sub> and 95% humidified atmosphere. Two different protocols was performed, first for  $3.5 \times 10^5$  cells/ml/well and secondly,  $1 \times 10^6$  cells/ml/well. Cells were stimulated with four different allergens, such as: *Lactobacillus rhamnosus* GG (LGG), Lipid A (LA/LPS/*E.coli*), Lipopolysaccharide (LPS/*E.coli*) and *Acinetobacter lwoffii* F78 (*Ac. lwoffii*) at three different concentrations of 10, 50, 100 ng per 1ml cell suspension, at incubation time of 24 and 48 hours. Afterwards, mRNA expression of TLR, cytokine and chemokine analysis was performed by Quantitative Reverse Transcriptase – PCR (qRT-PCR) method, using LightCycler System (Roche).



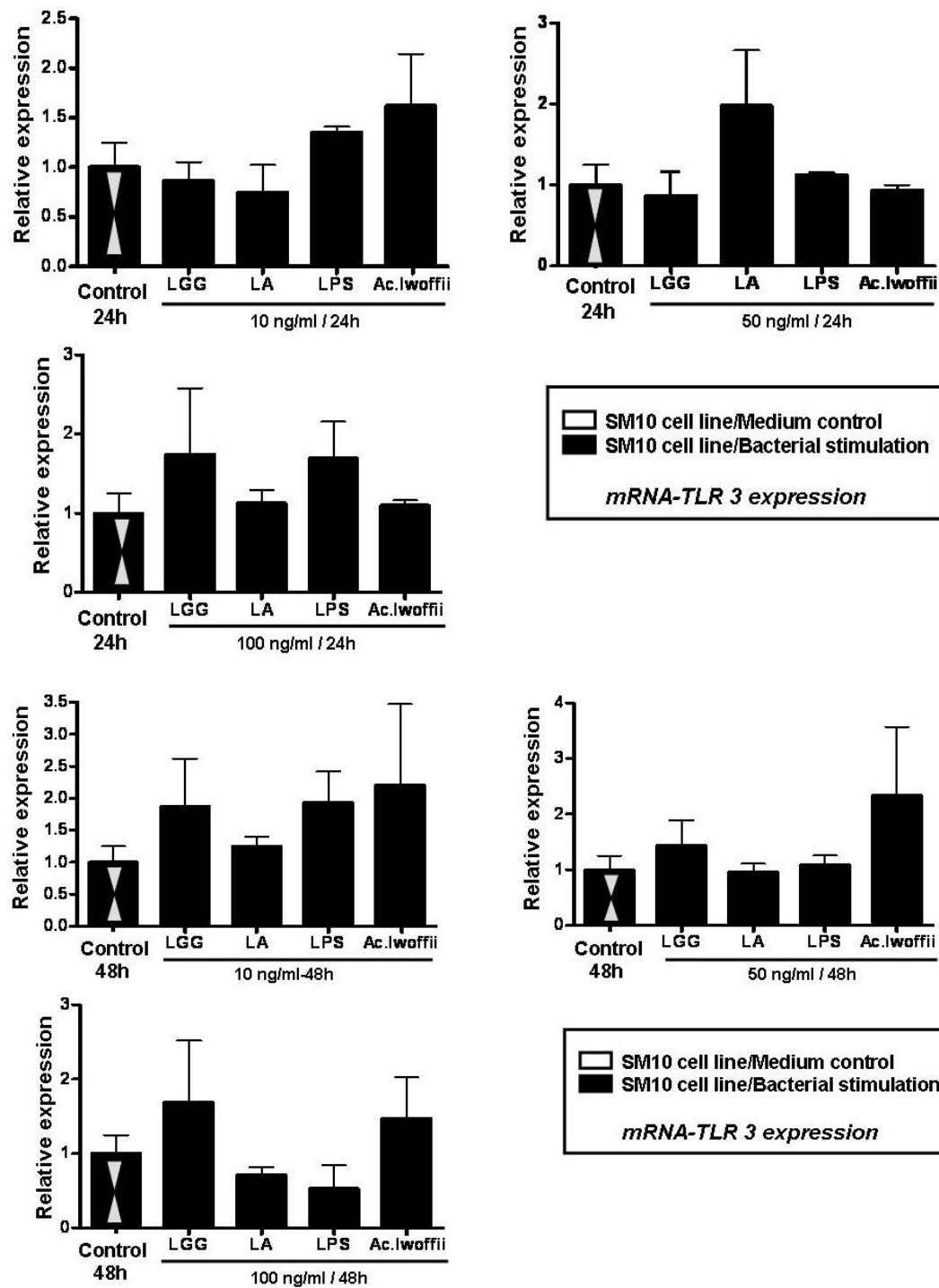


**Fig.4.1.2.1a SM-10 cell line, Trophoblast Stem Cells (TSCs) - mRNA TLR1 expression after LGG, LA, LPS and Ac. Iwoffii in vitro stimulation with 10, 50, 100 ng/ml conc., for 24h and 48h.** Variation between control and Ac. Iwoffii in-vitro stimulated TSCs at 50ng/ml, after 24h (\* $P < 0.05$  vs. ctrl; shown are mean  $\pm$  SEM), but not after 48h.

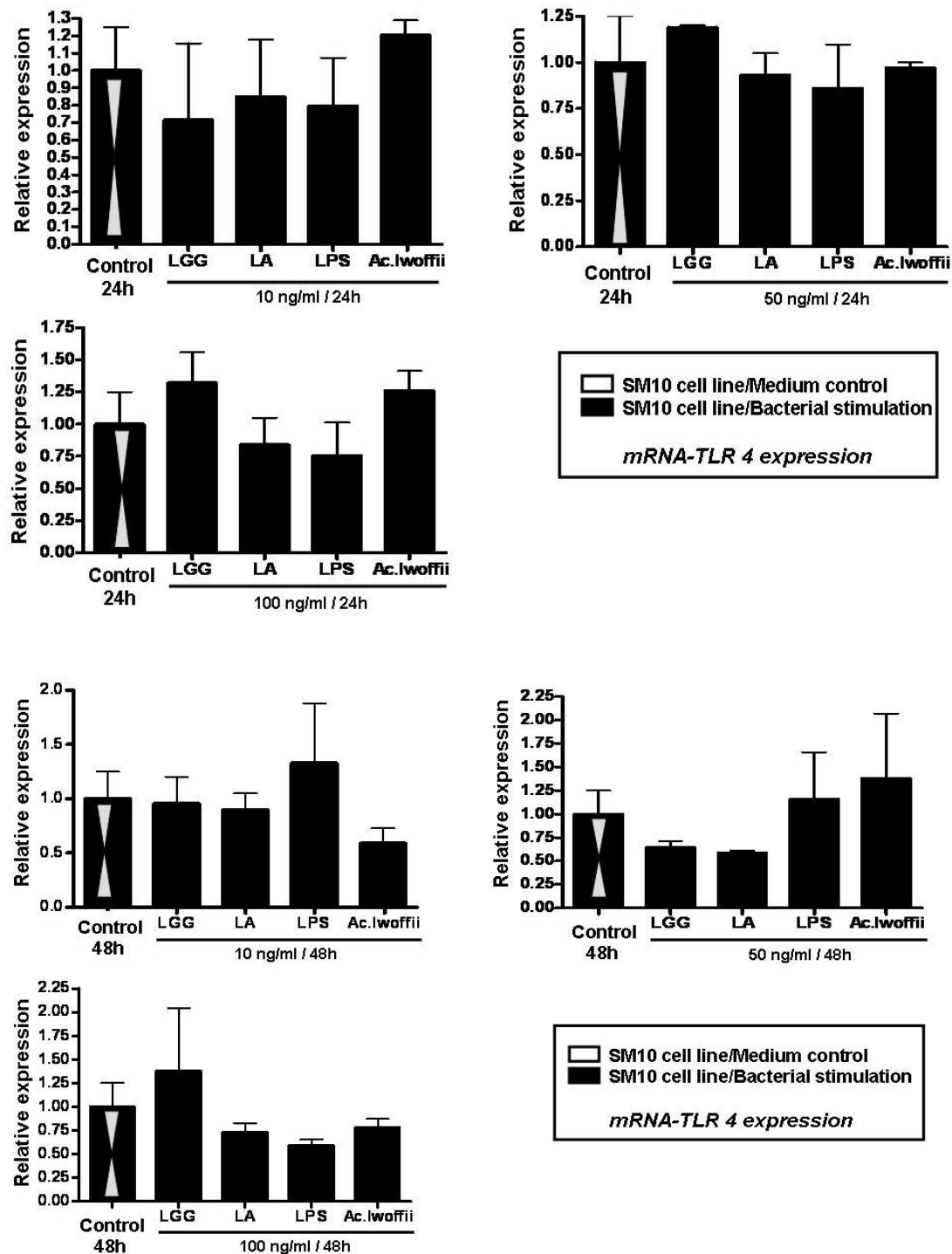


**Fig.4.1.2.1b SM-10 cell line, Trophoblast Stem Cells (TSCs) – state of mRNA TLR2 after LGG, LA, LPS and Ac. Iwoffii in vitro stimulation with 10, 50, 100 ng/ml conc., for 24h and 48h. No difference between control and in vitro stimulated TSCs.**

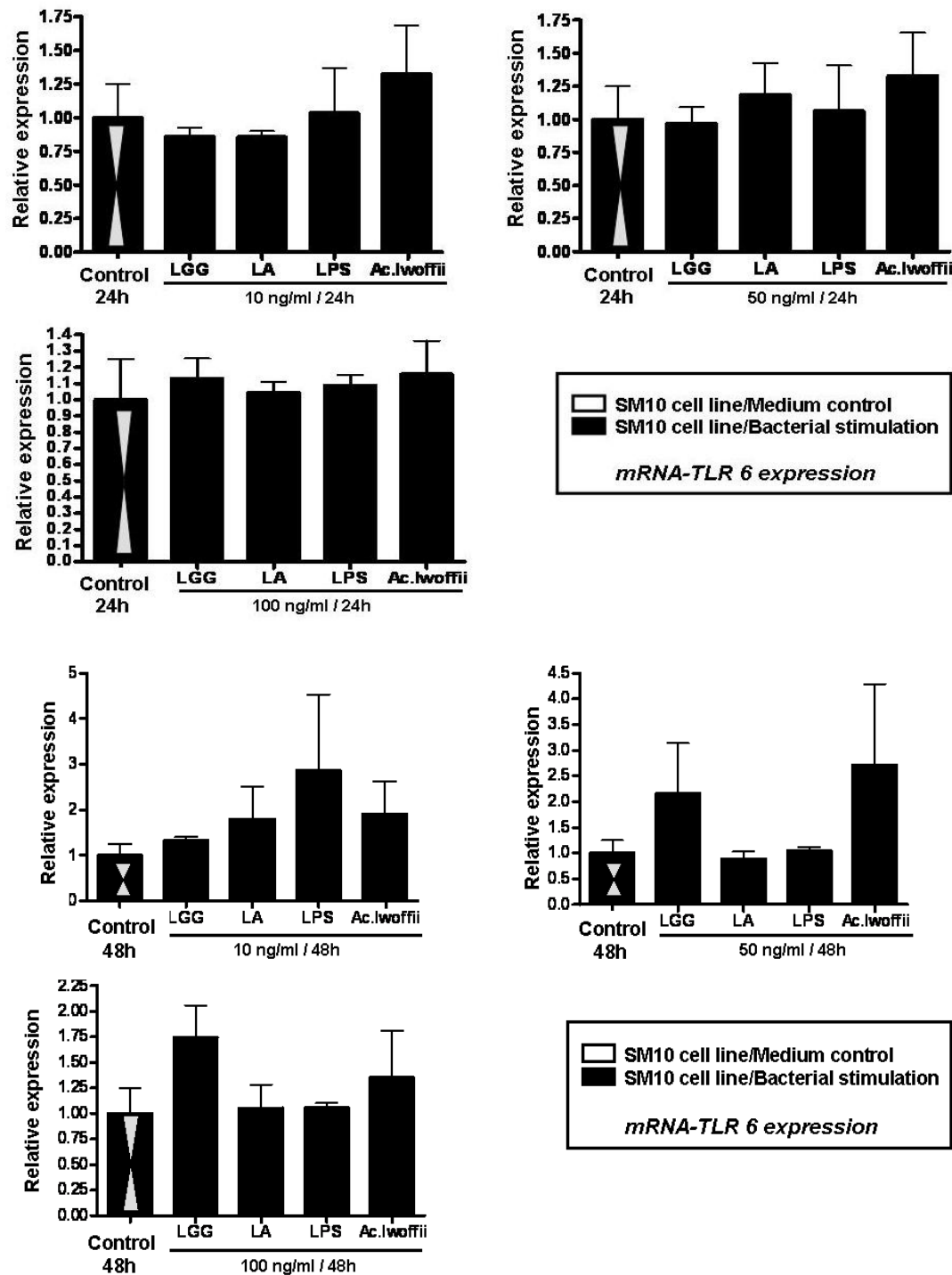




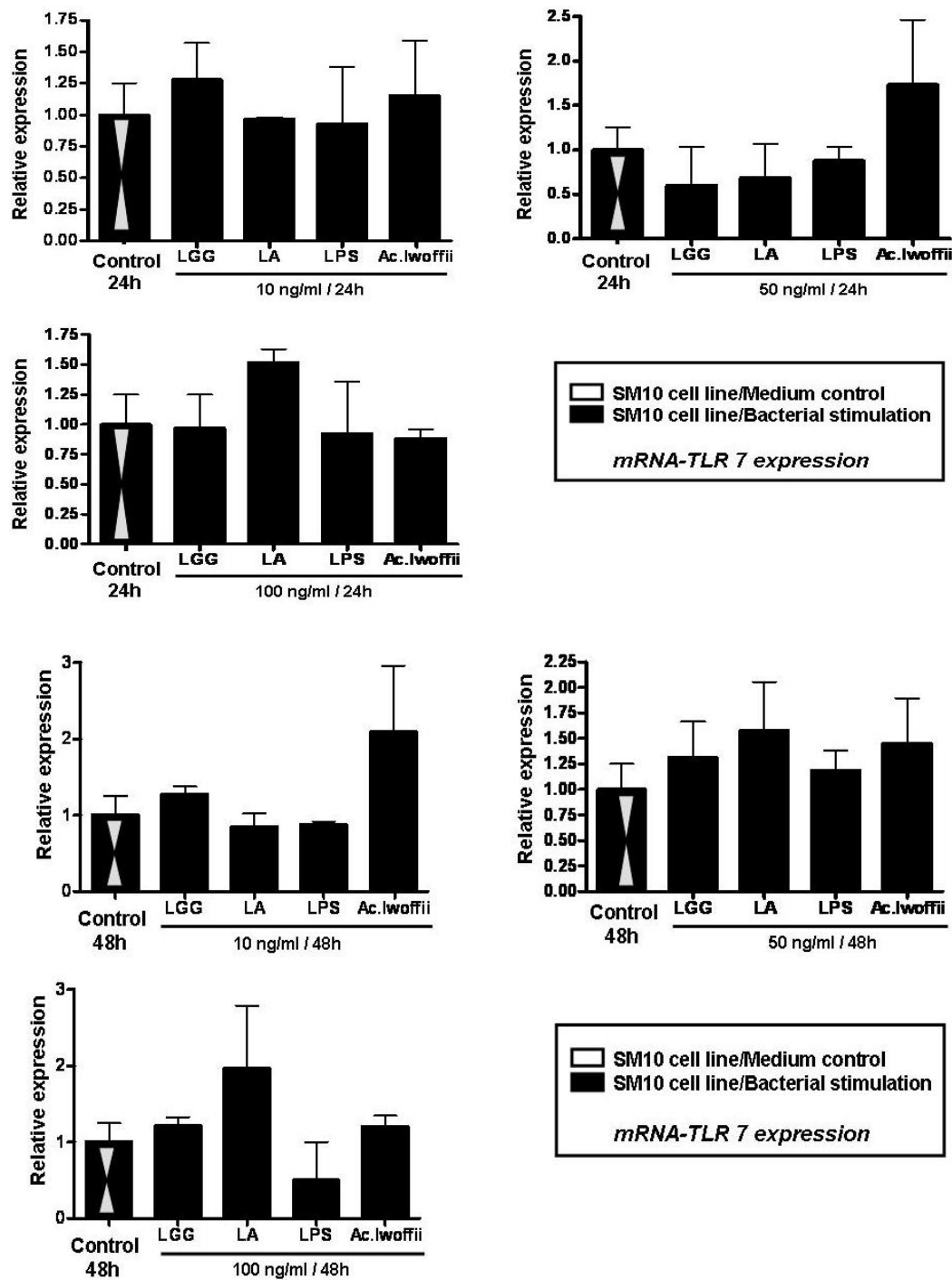
**Fig.4.1.2.1c SM-10 cell line, Trophoblast Stem Cells (TSCs) - state of mRNA TLR3 after LGG, LA, LPS and Ac. Iwoffii in vitro stimulation with 10, 50, 100 ng/ml conc., for 24h and 48h. No difference between controls and in vitro stimulated TSCs.**



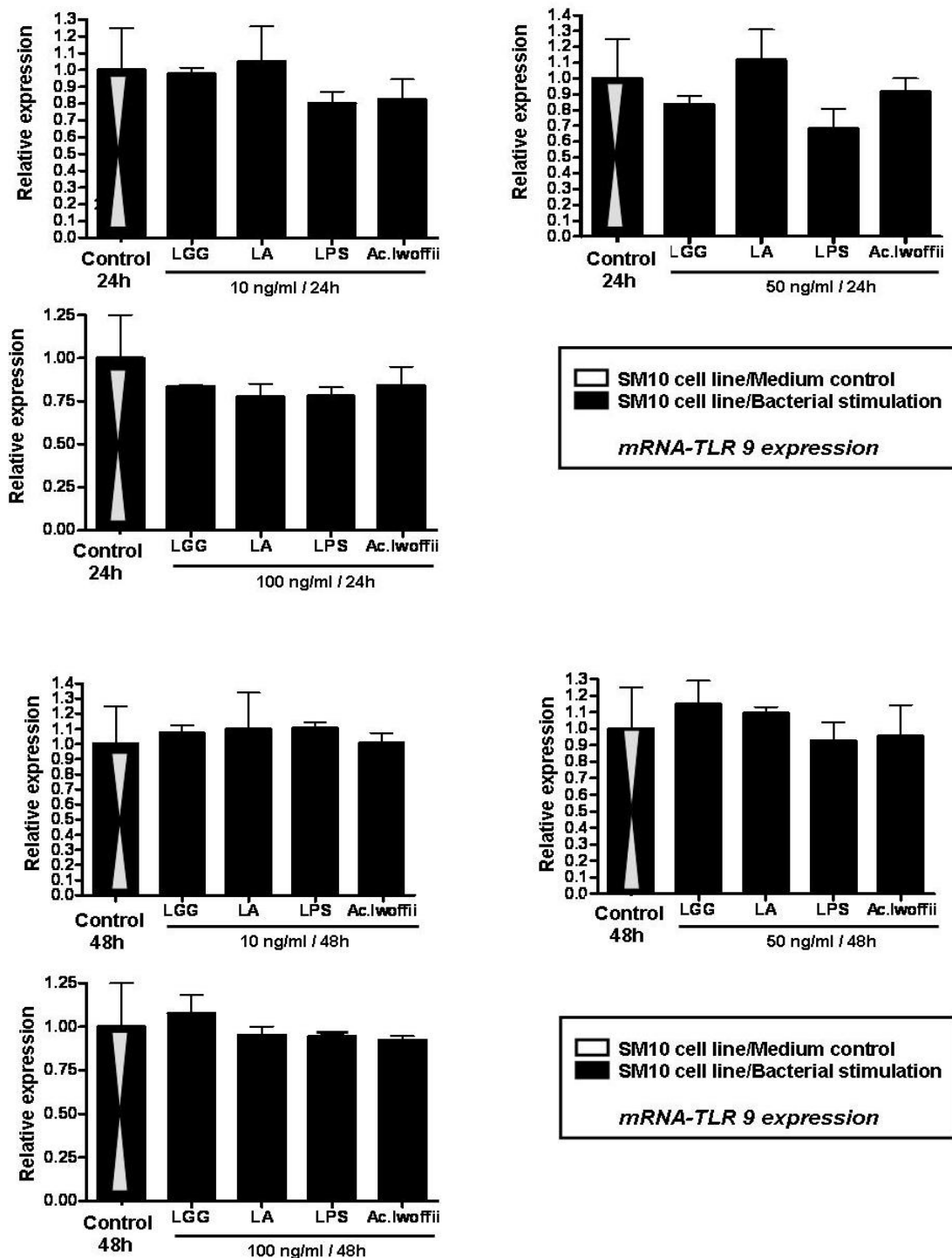
**Fig.4.1.2.1d SM-10 cell line, Trophoblast Stem Cells (TSCs) – state of mRNA TLR4 after LGG, LA, LPS and Ac. Iwoffii in vitro stimulation with 10, 50, 100 ng/ml conc., for 24h and 48h. No difference between controls and in vitro stimulated TSCs.**



**Fig.4.1.2.1e SM-10 cell line, Trophoblast Stem Cells (TSCs) – state of mRNA TLR6 after LGG, LA, LPS and Ac. Iwoffii in vitro stimulation with 10, 50, 100 ng/ml conc., for 24h and 48h. No difference between controls and in vitro stimulated TSCs.**



**Fig.4.1.2.1f SM-10 cell line, Trophoblast Stem Cells (TSCs) – state of mRNA TLR7 after LGG, LA, LPS and *Ac. Iwoffii* in vitro stimulation with 10, 50, 100 ng/ml conc., for 24h and 48h. No difference between controls and in vitro stimulated TSCs.**

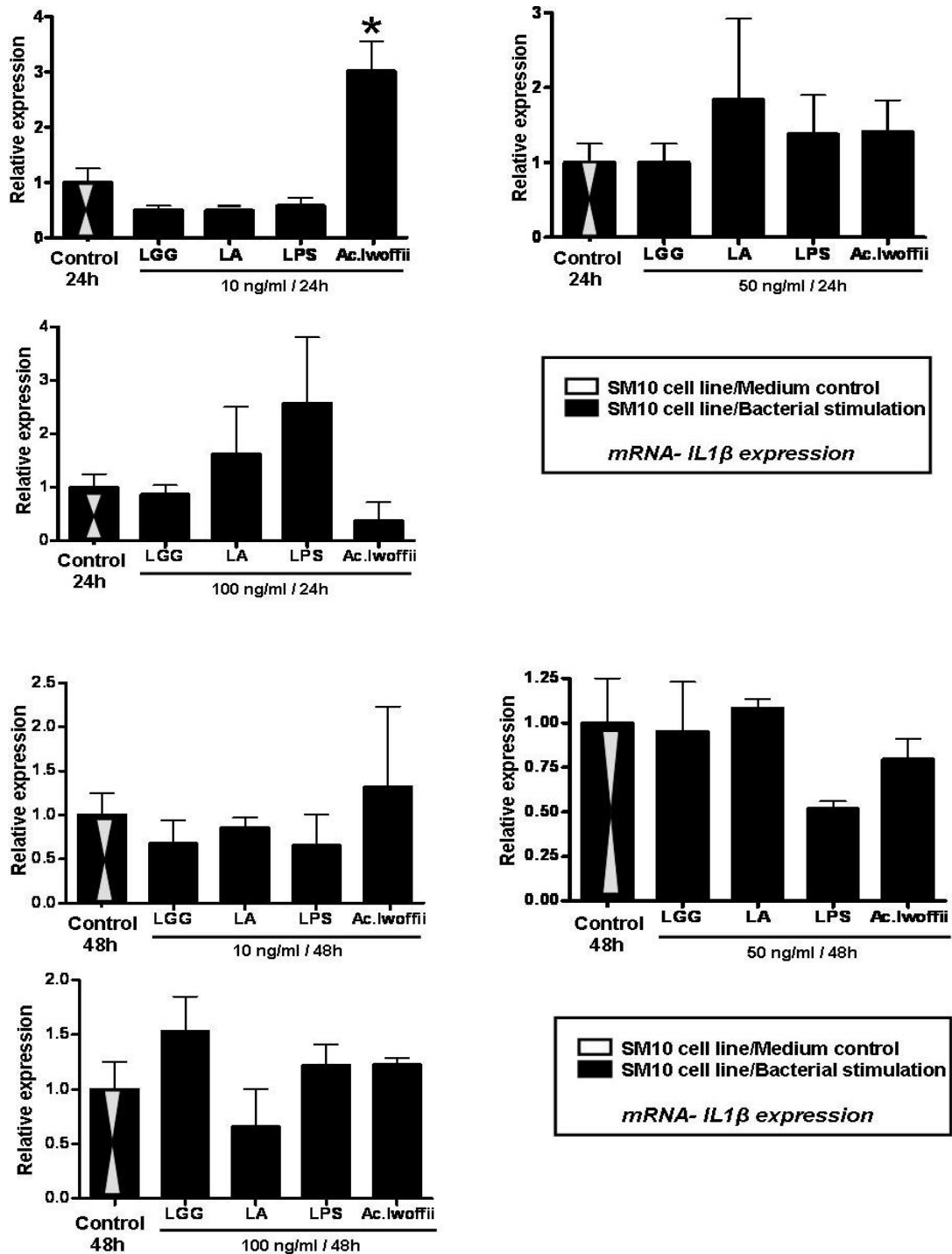


**Fig.4.1.2.1g SM-10 cell line, Trophoblast Stem Cells (TSCs) – state of mRNA TLR9 after LGG, LA, LPS and Ac. Iwoffii in vitro stimulation with 10, 50, 100 ng/ml conc., for 24h and 48h. No difference between controls and in vitro stimulated TSCs.**

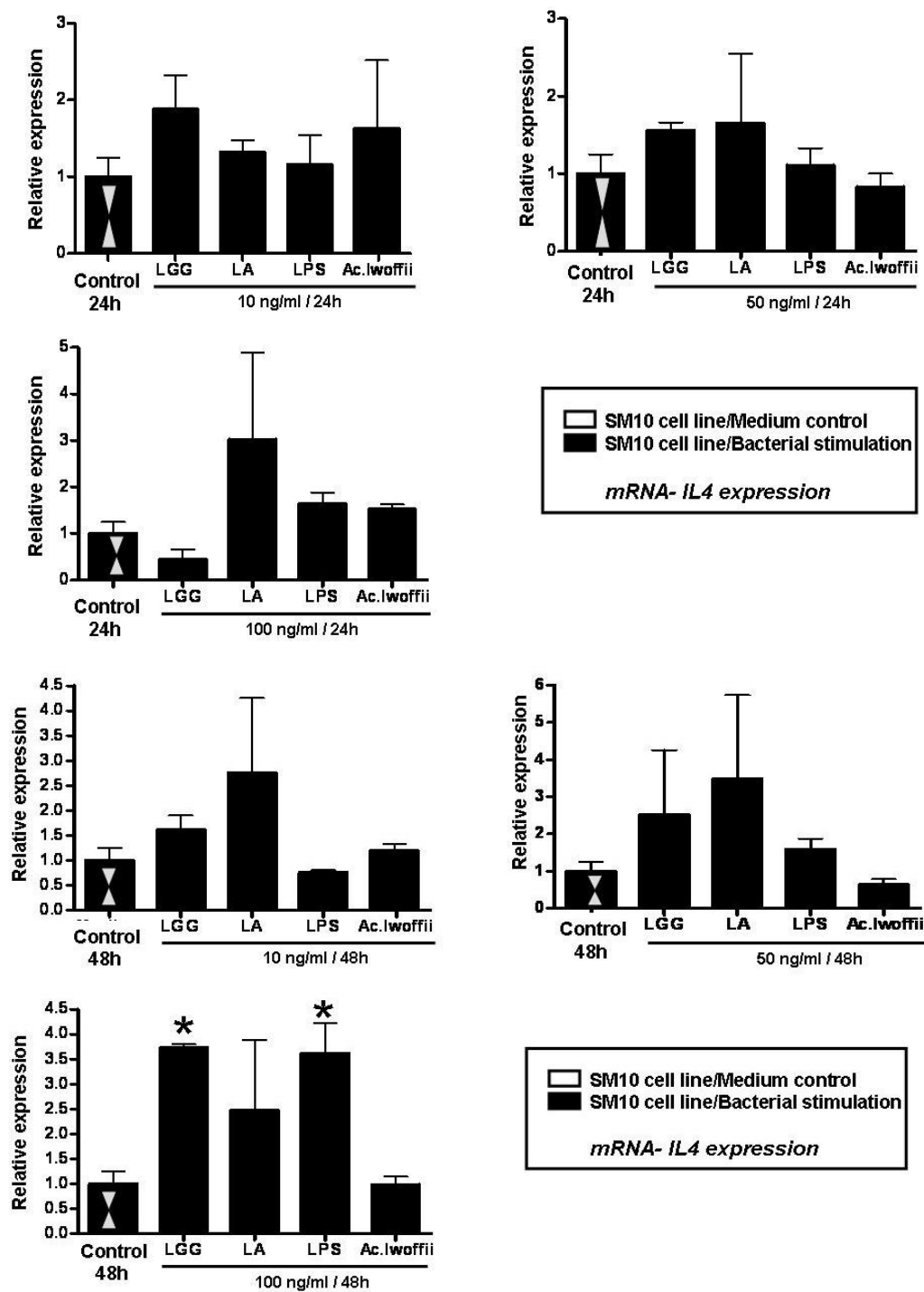
In vitro stimulated Trophoblast Stem Cells (TSCs) do not significantly express mRNA TLR, but mRNA TLR1 after 24 hours at concentration of 50 ng Ac.Iwoffii/1ml cell suspension. Thus, we performed comparative experiments between uncommitted trophoblasts stem cells and dedifferentiate trophoblasts (term trophoblasts), which demonstrated that TSCs need the attendance of growing and dedifferentiation factors, such as cytokine and chemokine (see Introduction chapter), being able to develop themselves and to complete their functions in maintenance the incomprehensible mechanisms of pregnancy.

#### **4.1.2.2 Trophoblast stem cells analysis: mRNA Th1/Th2 cytokine and chemokine expression after bacterial restimulation in culture**

The cytokines mRNA levels diverge from stimulated group to another. IL-1 $\beta$  and IL-4 expression is up-regulated in Ac. Iwoffii supplemented group; respective LGG/LPS stimulated TSCs *versus* controls. Trends include an inverse relationship in stimulated cells, in T<sub>H</sub>2 cytokines such as IL-10, while mRNA IFN $\gamma$  and TNF $\alpha$  have not been detected. Furthermore, the T<sub>H</sub>1/T<sub>H</sub>2 cytokines levels deliberated by ELISA shown a dramatically suppression IL-4 and IL-5. The IFN $\gamma$  and TNF $\alpha$  were not detected at all. Again, a comparison between both experiments, mRNA cytokines expression in TSCs and term trophoblasts, revealed us their behaviour and promising functions. The IL-1 $\beta$ , a pro-inflammatory cytokine is detected in term trophoblasts as well. Its principal effects similar to those TNF, is to attract leucocytes/endothelial adhesion molecules and inducing acute-phase proteins, or lymphocyte co-stimulation phagocyte activation, besides promoting the prostaglandin synthesis and tissue remodelling. Moreover, IL-4 is a T<sub>H</sub>2 cells released cytokine, which plays a key role in pregnancy, mediating the activation and division of B cells and promotes class switch IgG1 and IgE.



**Fig.4.1.2.2a SM-10 cell line, Trophoblast Stem Cells (TSCs) - mRNA IL-1 $\beta$  expression after LGG, LA, LPS and Ac. Iwoffii in vitro stimulation with 10, 50, 100 ng/ml conc., for 24h but no difference between controls and in vitro stimulated TSCs, after 48h. (\* $P < 0.05$  vs. ctrl; shown are mean  $\pm$  SEM).**



**Fig.4.1.2.2b SM-10 cell line, Trophoblast Stem Cells (TSCs) - mRNA IL-4 expression after LGG, LA, LPS and Ac. Iwoffii in vitro stimulation with 10, 50, 100 ng/ml conc., for 24h and 48h.** No difference between controls and in vitro stimulated TSCs after 24h, but after 48h with LGG and LPS in vitro stimulation (\* $P < 0.05$  vs. ctrl; shown are mean  $\pm$  SEM).



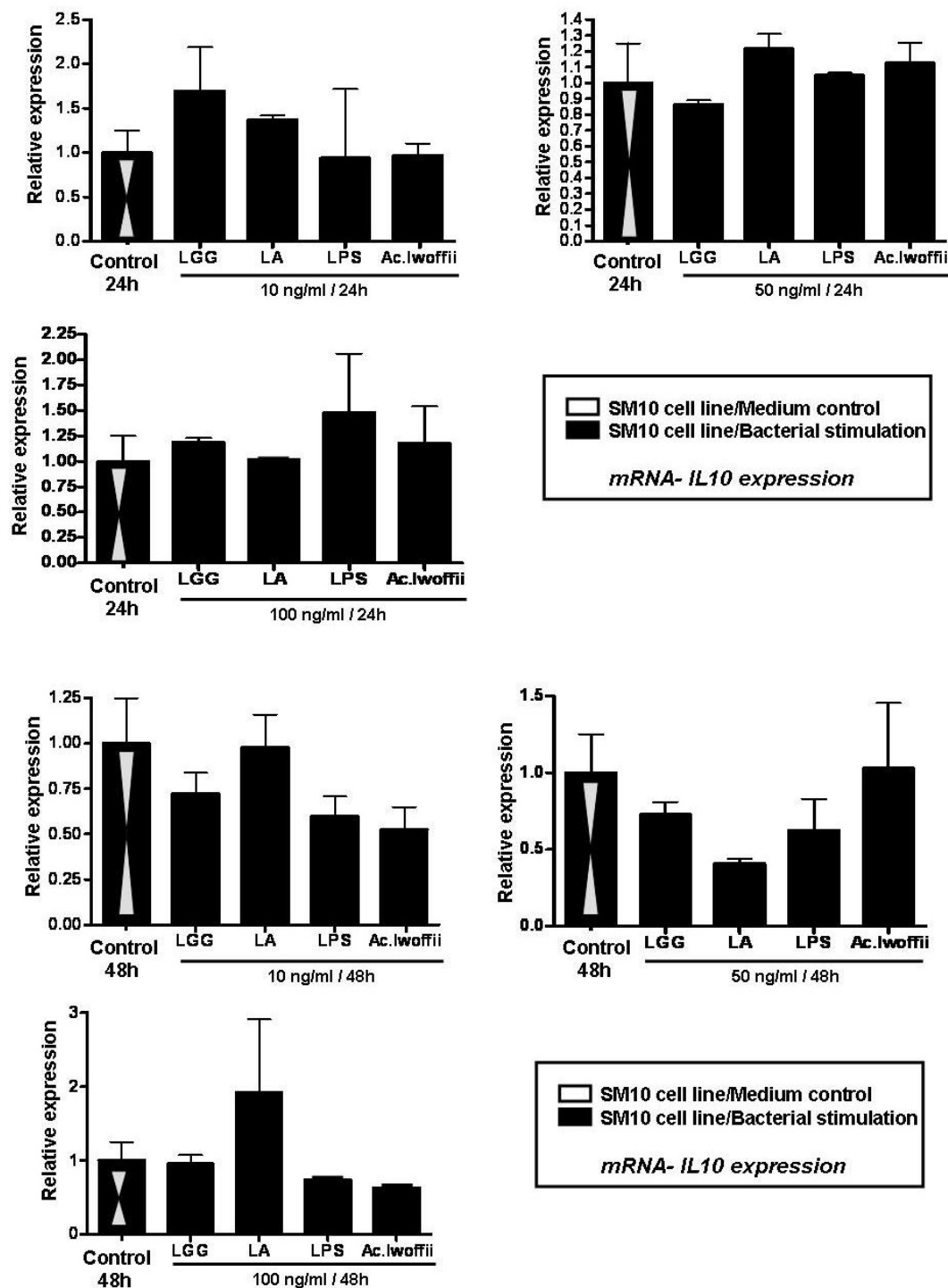


Fig.4.1.2.2c SM-10 cell line, Trophoblast Stem Cells (TSCs) – state of mRNA IL-10 after LGG, LA, LPS and Ac. Iwoffii in vitro stimulation with 10, 50, 100 ng/ml conc., for 24h and 48h. No difference between controls and in vitro stimulated TSCs.

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An essential aspect of the trophoblasts's phenotype in mouse or human (epithelial-like cell), they do not act absolutely like immune cells, but partially. They interact with lymphocytes through cross-talking mechanisms, appear to be like *sentinels* against immune attack to bacterial activated lymphocytes, using TLR or alternative pathways, maintaining the  $T_H1/T_H2$  balance pregnancy.

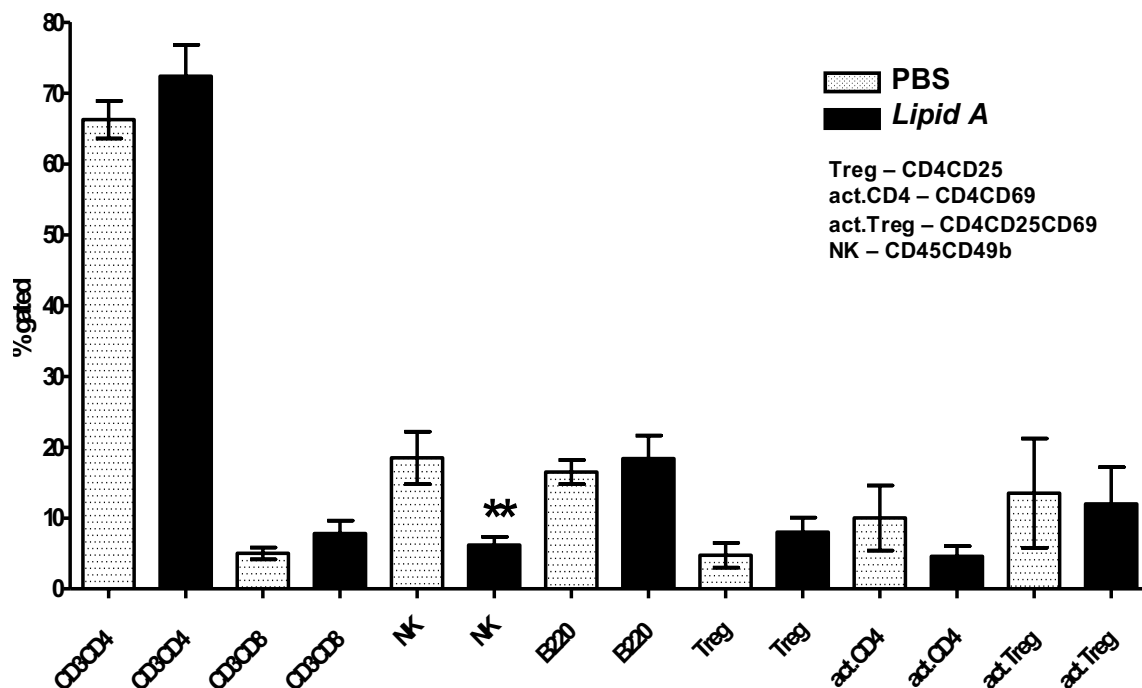
#### **4.1.3 Placental immune cells isolation and characterisation by Magnetizing-assesed Cell Sorting (MACS) and Flow Cytometry (FACS) Systems**

The function of the immune system is to protect the body from damage caused by bacteria, viruses, fungi and parasites. This defensive function is performed by leucocytes and a number of accessory cells (eosinophil and basophil granulocytes, mast cells, plateles and antigen-presenting cells), which are distributed throughout the body, but particularly are found in lymphoid organs including the bone marrow, thymus, spleen, lymph nodes and mucosa-associated lymphoid tissues (MALT). Lymphoid organs are strategically placed to protect different areas of the body from infection. Cells migrate between these tissues via the bloodstream and lymphatic system. Thus, they interact with each other to generate coordinated immune responses aimed at eliminating pathogens or minimizing the damage they cause. During the menstrual cycle, the number of  $CD45^+$  leukocytes increases pre-menstrual from 10-15% to 20-25% of all endometrial cells (Markert, 2005). During the first trimester of pregnancy, the number increases further and approximately 70% of immune-competent decidual cells are  $CD45^+$  leukocytes. The predominant type of these cells are transient, pregnancy-associated uterine natural killer cells – uNKs - (46%), followed by macrophages (19%) and T cells (8%), mainly  $CD8^+$ T cells.

As shown in various knockout animal models, most classes of immune cells are crucial for successful pregnancy, but their function and way of action are modified compared with leukocytes from peripheral blood, other tissues of inflammation areas. It may be suggested that such modifications are regulated

on the signal transduction level, the JAK/STAT pathway. Recent data, suggests that this system is involved in the differentiation of T helper cells into T<sub>H</sub>1 and T<sub>H</sub>2 subsets. Many cytokines signal via this pathway and control it. Also, the production of numerous cytokines and other factors depends on JAK/STAT signalling. Therefore, could be suggested that the regulation of the JAK/STAT system is essential for the development and maintenance of the immunological balance in the decidua and placenta necessary for successful pregnancy (Markert, 2005). In this regard, we speculate that placenta could plays an important role as a *fetal lymphoid organ* in pregnancy, initially protecting the fetus against rejection or early abortive process, suppressing the maternal immune system and subsequent, soon after birth and early childhood, against allergies such as asthma associated. In this regard, we established an experimental protocol, where the total CD45<sup>+</sup> placental cells are characterised, prior using a positive selection method by Magneting-assesed Cell Sorting (MACS), followed by Flow Cytometry System (FACS) analysis. Placental MNCs were isolated in a similar way as splenic MNCs, described in Materials and Methods chapter. We used an immunofluorescent staining protocol for cell surface molecules with specific markers such as, CD45 for total number of leukocytes-common-antigen (LCA), CD3, CD4, CD8 for the T cells, respective T cell subsets (helper T cells, cytotoxic T cells, memory T cells), CD49b for natural killer cells (NK), CD4CD25 for regulatory T cells, CD69 for activating T<sub>regs</sub> and NKs, CD11b and CD11c for macrophages and dendritic cells (DCs). The cells were harvested and stained with diverse fluorochrome-conjugated mouse antibodies. Cells are analyzed by means of Flow Cytometry on a FACSCalibur using the CellQuest Program (Becton Dickinson, San Jose, California). Data analysis is performed with the WinMDI 2.8 Software developed by Joseph Trotter (Scripps Institute, La Jolla, California). The idea behind was to characterise the main immune cell classes (T, B, NKs, DCs) and some subclasses related to CD45<sup>+</sup> cells (regulatory T cells, activated T cells and activated T<sub>regs</sub>). They cross placenta during pregnancy and contribute in maintenance of the feto-maternal immunity balance, within specific circumstances. We preferred to investigate the LA supplemented group, since

the Lipid A is the active component of LPS/E.coli, and moreover the postnatal data from our Laboratory and other groups demonstrated that Gram<sup>-</sup> bacteria and/or LPS in certain concentration are able to modulate allergic reactions. Here, we observed only a trend in induction of CD3<sup>+</sup>CD8<sup>+</sup> T cells, but a significant suppression of NK cells in LA supplemented group versus control.



**Fig.4.1.3 Comparative characterization of placental immune cells – APC cells, T and T<sub>reg</sub>, Mφ, NKs in PBS and LA supplemented groups.** FACS analysis demonstrated a significant decrease in placental NK cells of the LA (LPS/E.coli, Gram<sup>-</sup> bacterium) treated group versus controls, while placental histology does not reveal any differences between groups (\*\*P< 0.01 vs. ctrl; shown are mean ± SEM; n=6 mice/study group, n=1 placenta/mouse female).

The suppression process, the cellular basis for this type of immunoregulation implicates some or all of these well known mechanisms: (i) a specific cytostatic action of CD8<sup>+</sup> Tc cells; (ii) passive blocking of lymphocyte activation by sequestering essential cytokines required for cell division; (iii) secretion of immuno-suppressive molecules such as prostaglandins or TGF-β; (iv) an immunoregulatory effect caused by the local production of specific sets of cytokines; (v) induction of clonal anergy due to the T suppressors (CD3<sup>+</sup>CD8<sup>+</sup>T

cells) supplying an activation signal but not the required co-stimulatory signals or cytokines. NK cells, the large granulated lymphocytes, are the dominant decidual cell population from the first stage of pregnancy. After blastocyst implantation and decidualization, dNK cells are activated, they produce IFN $\gamma$ , perforin and other molecules, including angiogenetic factors. Therefore, they can control trophoblasts invasion through their cytotoxic activity, and also initiate vessel instability and remodelling the decidual arteries to increase the blood supply to the placental bed. Alternatively, they are involved in cytokine-mediated immunoregulation of the maternal immune response producing T<sub>H</sub>2- and T<sub>H</sub>3-type cytokines, which result in placental development, in local immunosuppression, respectively in immunomodulation.

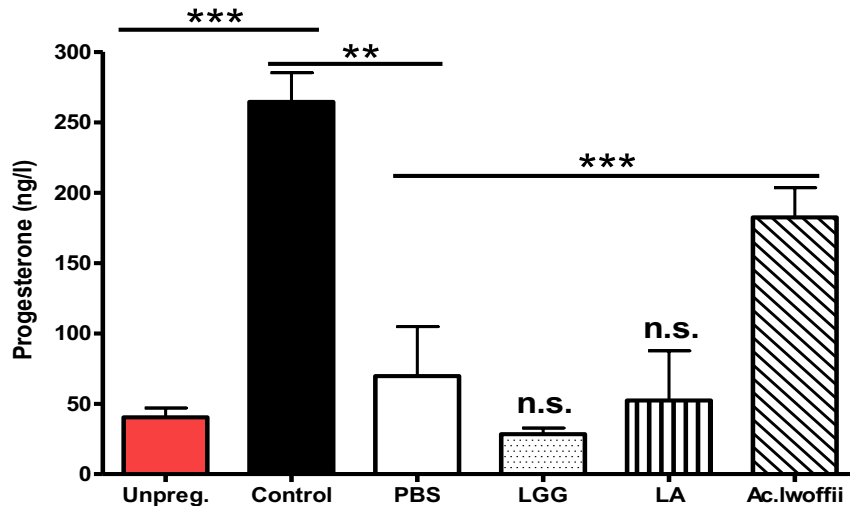
#### **4.1.4 Maternal blood serum and amniotic fluid analysis: measurement levels of steroid hormones, cytokine and chemokine by ELISA and CBA kit**

Of particular interest are the interactions between the reproductive system and the immune system. The reproductive system is unique in that its primary role is to assure the continuity of the species, while the immune system provides internal protection and thus, facilitates continue health and survival. The *modus operandi* of these two morphologically diffuse systems involves widely distributed chemical signals in response to environmental input, and both systems must interact for the normal functioning for each. While the major focus of reproductive-immune research has historically been with mammals, and has provided substantial insight into the interactions between these physiological systems, comparative studies offer unique perspectives. It is well known that reproductive hormones have been implicated in the development, as well as modulation, of the immune system (Mann, 1994). Although the effects of estrogen and progesterone on white blood cells are diverse, they have demonstrated an overall inhibitory effect on leukocyte proliferation. Also, these steroids have been shown to exert dose and time-dependent effects on

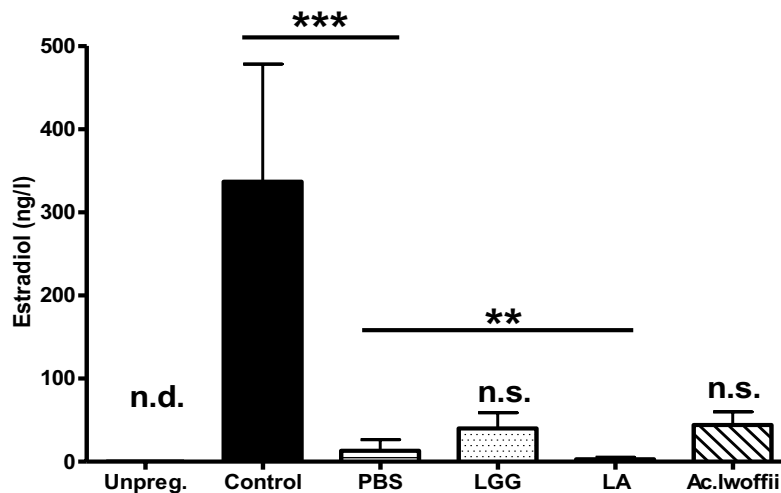
apoptosis in mononuclear cells, T- and B-lymphocytes, neutrophils, and cancer cells. Of great potential importance, interactions between the reproductive hormones and the immune system have been investigated due to correlations in autoimmune diseases. Thus, women experience improvement and amelioration of symptoms in these diseases during pregnancy, when hormone levels increase (Beagley, 2003). Other researchers provided evidence that the immune system is suppressed by reproductive effort in that antibody response to immune challenge and parasite resistance are reduced, respectively. Regarding, an evolutionary trade-off between reproductive fitness and immune defence has been hypothesized due to T cells having an overall immunosuppressive effect (Folstad and Karter, 1992). For instance, T helper 1 ( $T_H1$ ) cells secrete proinflammatory cytokines, such as  $TNF\alpha$  and  $IL-1\beta$ , and promote cell-mediated immune responses. T helper 2 ( $T_H2$ ) cells secrete cytokine that promote antibody production. Progesterone promotes the development of a  $T_H2$  response during pregnancy, which antagonizes the emergence of  $T_H1$  cells. During pregnancy, immunoregulatory system must operate locally at the placental interface, being reversible to preserve the systemic immune competence of the mother. Several mechanisms involving steroids, especially progesterone, may contribute to fetal-maternal protection, including altered expression of MHC class I proteins in fetal tissue, altered T cell subsets, reduced number of NK cells, or elaboration of immunosuppressive factors (Pepe, 1995).

In our prenatal study, we monitored the development of maternal and fetal immunity during bacterial exposure, respective after neonate's asthma induced quantifying by ELISA the most important steroids's levels, such as Progesterone and Estradiol from maternal mouse blood serum and amniotic fluid. The experimental protocol has been established for five different groups of mouse females: (i) Nonpregnant/Nonsupplemented (only blood serum), (ii) Pregnant/nonsupplemented used as Control for all supplemented groups, (iii) Pregnant/PBS (control group), (iv) Pregnant/LGG, (v) Pregnant/Lipid A, (vi) Pregnant/Ac. Iwoffii supplemented groups. Our observations reveal that in serum the progesterone level shown a significantly up-regulation only in the Ac. Iwoffii

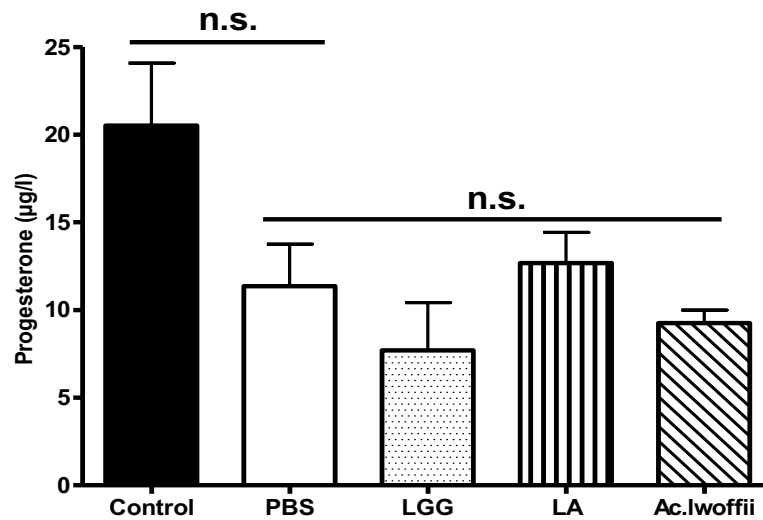
supplemented females, while the estradiol level is suppressed in LA supplemented group, related to control / PBS groups.



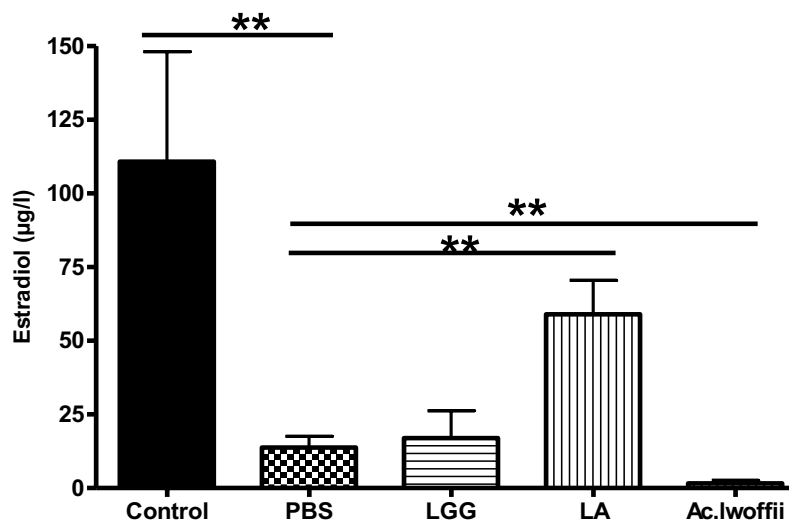
**Fig.4.1.4a Maternal serum steroid hormone, progesterone - quantification by ELISA method.** Progesterone is high induced in control pregnant group vs. un-pregnant and all other supplemented pregnant groups. Also, Ac. lwoffii supplemented group illustrate an up-regulation in serum progesterone level in comparison with PBS (ns-not significant; \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. Control and PBS pregnant females; shown are mean  $\pm$  SEM;  $n = 5$  mice/study group)



**Fig.4.1.4b Maternal serum steroid hormone, Estradiol - quantification by ELISA method.** Estradiol is high induced in control pregnant group versus un-pregnant (could not be seen with the implemented limit detection), and all other supplemented pregnant groups. Also, it shows a low quantification level in Lipid A supplemented females related to PBS group (ns-not significant; nd-not detectable; \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. Control and PBS supplemented pregnant females; shown are mean  $\pm$  SEM;  $n = 5$  females/study group).



**Fig.4.1.4c** Schematically illustration of amniotic fluid maternal steroid hormone, such as **Progesterone** quantified by ELISA. Progesterone level was not affected after bacterial supplementation, related to PBS, respective Control groups (ns-not significant; shown are mean  $\pm$  SEM; n= 5 females/study group).

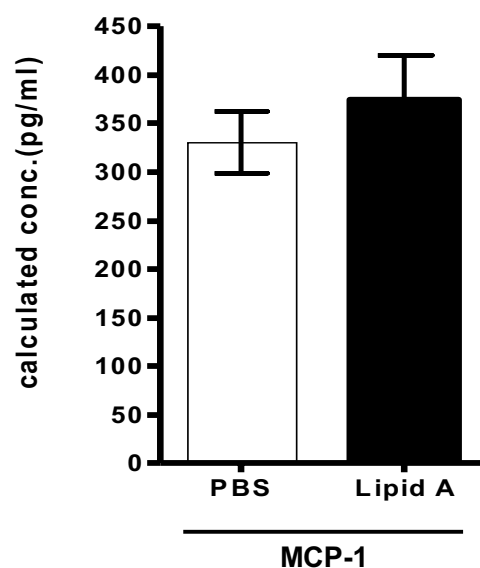


**Fig.4.1.4d** Schematical illustration of amniotic fluid maternal steroid hormone, such as **Estradiol**. Estradiol levels shown an inverse relationship between LA, Ac. lwoffii supplemented pregnant females related to PBS, respective Control group, at pregnancy's term. This represent signal of hormonal variation given by pregnancy state related with bacterial persuade in fetal immunity (\*\*P< 0.01 versus PBS/Control groups; shown are mean  $\pm$  SEM; n= 5 females/study group)



In addition to the lack of classical MHC class I and II antigens at the trophoblast sides the cytokines play an important role at the maternal-fetal interface. Different cytokines regulate the immunological processes with an emphasis on the  $T_H1/T_H2$  balance. Chemokine (C-C motif) ligand 2 (CCL2) is a small cytokine belonging to the CC chemokine family that is also known as monocyte chemoattractant protein-1 (MCP-1). CCL2 recruits monocytes, memory T cells, and dendritic cells to sites of allergen reaching tissue. CCL2 causes the degranulation of basophils and mast cells, an effect potentiated by high concentration of IL-3 and/or other cytokines.

In contrast to the peripheral blood, there is a certain composition of immune competent cells at the feto-maternal interface. There are many different interactions between the endocrine and immunological system, where the steroids hormones modulate the secretion of certain cytokines and vice versa.



**Fig.4.1.4e Amniotic fluid MCP-1 chemokine is significantly induced in LGG supplemented females, but not in the other groups.** Measurements are performed by CBA kit (\*\* $P < 0.01$  vs. PBS; shown are mean  $\pm$  SEM;  $n = 5$  females/study group).

Equivalent measurements are performed by CBA kit (followed the manufacturer description) either in blood serum and amniotic fluid for other cytokines, such as IL-4, IL-5, IL-10, IL-12p70,  $TNF\alpha$  and IFN $\gamma$ . The results reveal no significant

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difference between bacterial supplemented and control groups. Therefore, it is expected that relatively a slight perturbations of the  $T_H1/T_H2$  balance can have an impact, either decreasing or increasing a risk of later allergic and/or airway diseases.

#### **4.2 Acute asthma model: perinatal studies with experimental mouse model. Protective effects on the offspring observed in development of early allergic diseases**

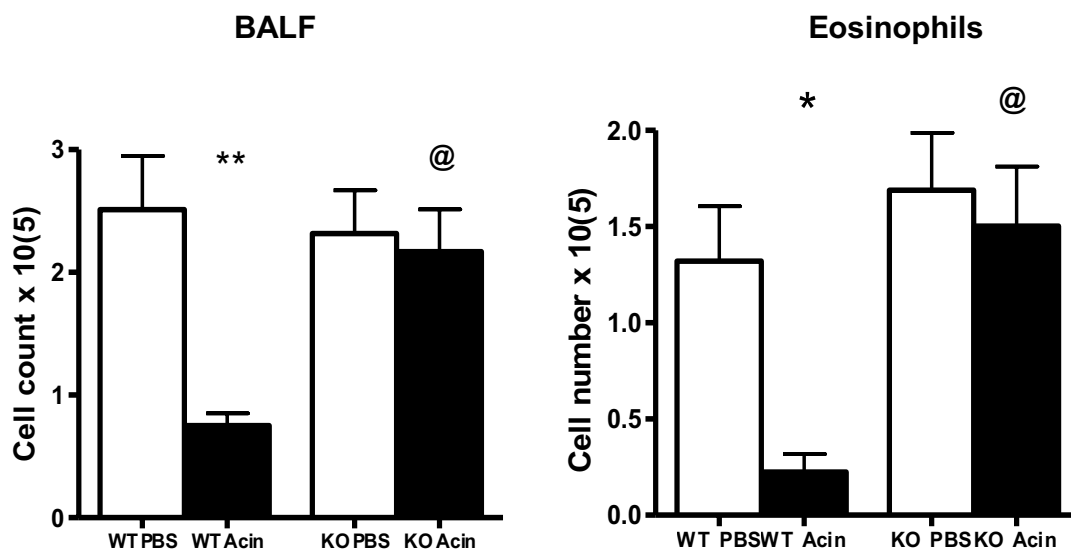
A large number of clinical investigations direct on human or experimental, on animal models provided strong evidence that due to an early-life contact with farm animals, cowsheds and/or consumption of products like raw milk might have an important effect on the development allergies in order to influence allergic response - protective mechanisms. Thus, data on intrauterine mouse model (Balb/c strain) and acute asthma animal model experimental design established in our laboratory (Blümer, 2007 & Debarry, 2007) support the *Hygiene hypothesis* which states that a farming environment rich in microbiologic structures might protect against the development of allergies. Thus, certain microbes present in farming regions of children living, act by promoting an immune deviation from  $T_H2$  to  $T_H1$  in the allergen – specific response. Because the innate immune system plays an essential role in the initial immune activation and modulates subsequent adaptive immune responses, we investigated in maternal tissues due to pregnancy period, where the existing pattern-recognition receptors (PRRs) like as Toll-like receptors (TLR) are involved through specific signalling pathways, in respect to applied bacterial supplementation. In consequence, the postnatal experimental part corroborates mostly with the prenatal data and reveals together a potential protective molecular mechanism against allergies, such as asthma associated. Therefore, in our supplementary experimental protocol, proving the TLR-mediated asthma defensive molecular mechanism, we used C57BL/6J strain female mice, 6-8 weeks old homozygote  $MyD88^{-/-}$ ,  $TLR^{-/-}$  ( $TLR2/3/4/7/9^{-/-}$ ),  $TNFP55^{-/-}$  versus wild type female mice. For mating are used

wild type male mice, C57BL/6J background, 6-8 weeks old. All groups received intranasal (i.n.) lyophilized *Acinetobacter lwoffii* ( $10^8$  CFU per application, reconstituted in a total volume of 50  $\mu$ l PBS), 10-8-6-4-2 days before conception and subsequently, the maternal supplementation continued every second day until term of pregnancy. Age-matched control animals received PBS. Later on after birth, asthma was induced in the young animals (heterozygote offspring MyD88<sup>+/-</sup>, TLR<sup>+/-</sup>, TNFp55<sup>+/-</sup> 4 weeks old). Offspring are sensitized twice to Ovalbumin (OVA) at the age of 28 and 42 days; intraperitoneal (i.p.) injections of 10  $\mu$ g OVA are applied (grade VI; Sigma, Diesenhofen, Germany), emulsified in 1.5 mg Al(OH)<sub>3</sub> (Pierce, Rockford, USA) in a total volume of 200  $\mu$ l. At days 48, 49, 50 the subgroups of offspring were placed one after other in a special Plexiglas chamber and exposed to aerosolized 1% (wt/vol) OVA solution (reconstituted in PBS), 20 minutes time exposure.

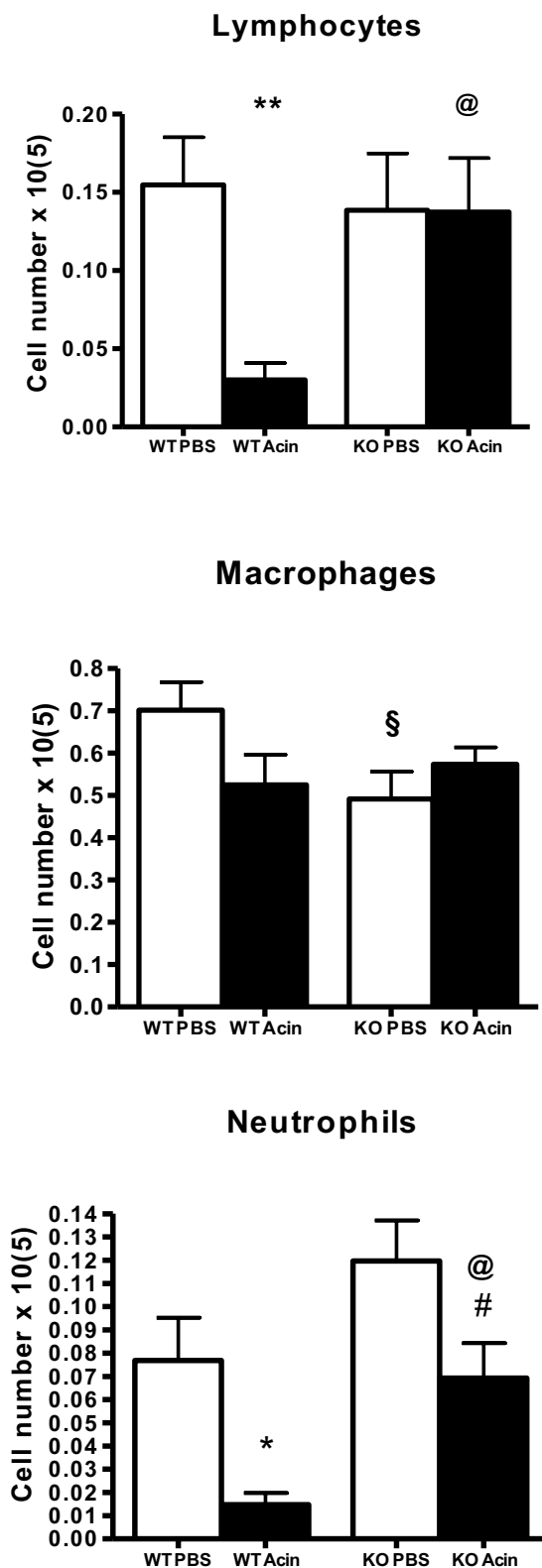
#### **4.2.1 The effect of maternal non-pathogenic bacterial exposure on offspring lung: airway hyperresponsiveness (AHR) and bronchoalveolar lavage fluid (BALF) analysis**

To prove whether the effects of prenatal exposure to non-pathogenic bacteria either Gram<sup>+</sup> and Gram<sup>-</sup> bacteria or components of Gram<sup>-</sup> bacteria, such as LA influences the development of experimental asthma, OVA-sensitized mice were aerosol allergen challenged for three times. Airway inflammation was investigated by analysing bronchoalveolar lavage fluid (BALF). BAL cellular composition is considered like: total number of leucocytes, number of eosinophilic cells, lymphocytes, macrophages and neutrophils. We observed that *Acinetobacter lwoffii* supplementation appears to act through maternal TLR, to reduce BALF cell - eosinophil and lymphocyte counts in asthmatic offspring. Furthermore, TLR 2/3/4/7/9<sup>+/-</sup> offspring mice have decreased numbers of BALF macrophages. Moreover, *Ac. lwoffii* action appears to be through maternal Toll-like receptors signalling pathway, but alternative pathways can be also present affecting BALF neutrophils. To observe the effects of maternal exposure to *Ac.*

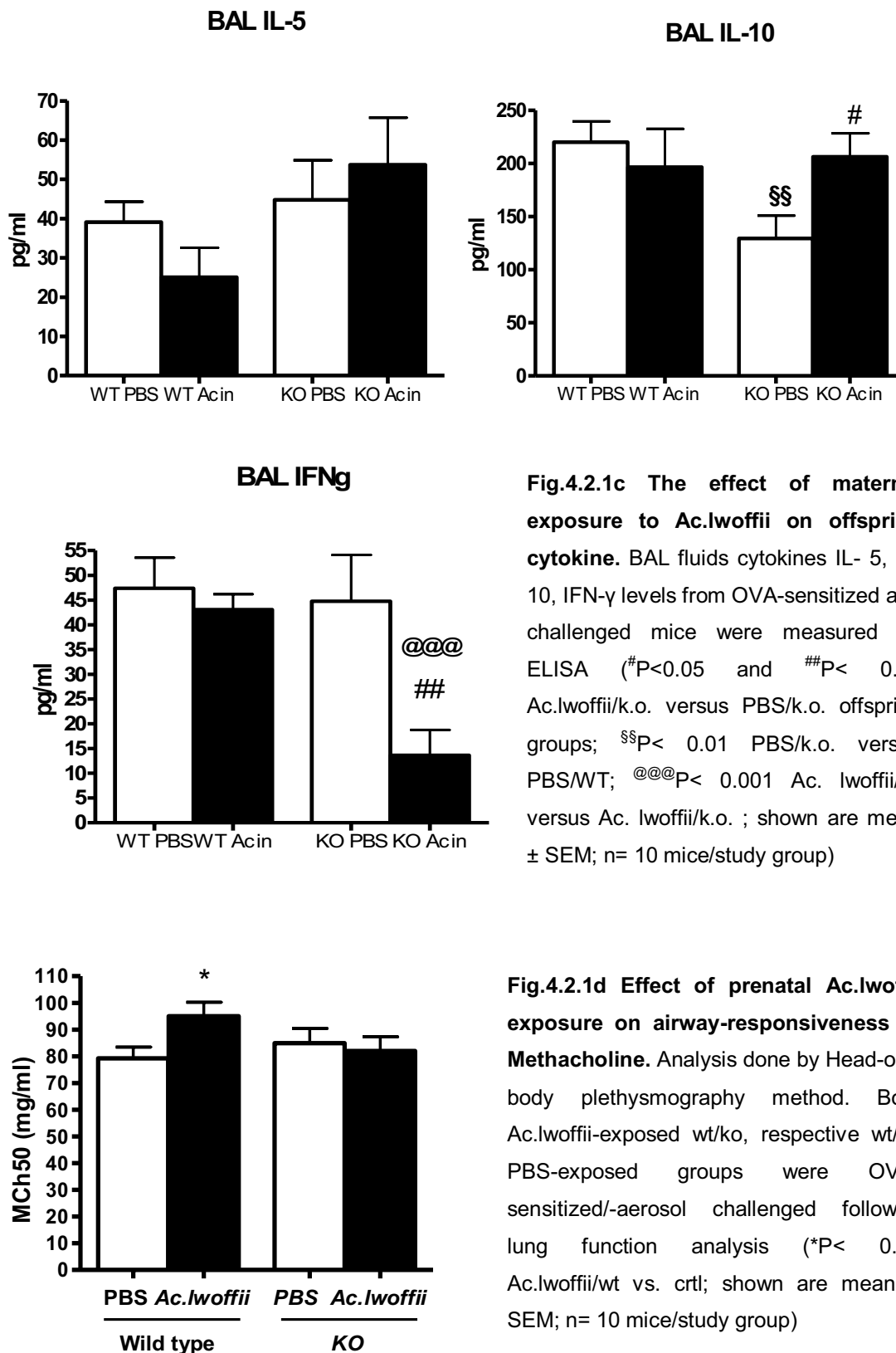
lwoffii on offspring cytokines and more, the course of immune response, BALF cytokines such as IL-10, IL-5, IFN $\gamma$  levels from OVA-sensitized and challenged mice were measured by ELISA method. Accordingly, in the absence of maternal TLR, Ac. lwoffii supplementation has an effect on asthmatic offspring BALF - IFN $\gamma$  levels. We observed a strong down-regulation of IFN $\gamma$  in (TLR $^{+/-}$  offspring Ac. lwoffii supplemented group) K.O./Ac. lwoffii in relationship with control deficientes (TLR $^{+/-}$  offspring PBS supplemented group) K.O./PBS and wild type wt/PBS and wt/Ac. lwoffii groups. The results reveal an opposite effect, respective an induction of T $_H$ 2 immunity in comparison with the phenotype of asthmatic offspring Balb/c strain, which reveal a T $_H$ 1 immunity – an up-regulation of BALF IFN $\gamma$  - acquired in our postnatal studies (Debarry, 2007). Also, TLR $^{+/-}$  offspring cover reduced BALF IL-10 production, but no difference seen in BALF IL-5 levels.



**Fig.4.2.1a Effect of prenatal exposure to Ac. lwoffii on airway inflammation in OVA - immunized and allergen challenged mice.** Wild-type and TLR $^{+/-}$  offspring mice, C57BL/6 background were OVA-immunized. Both of the wt/k.o. Ac. lwoffii-exposed, respective wt/k.o. PBS-exposed groups were OVA aerosol challenged followed by a lavage of the airways. Cellular composition of BALF, such as: total number of leucocytes and eosinophils (\*P< 0.05 and \*\*P< 0.01 Ac. lwoffii/wt versus PBS/wt; @P< 0.05 Ac. lwoffii/k.o. versus Ac. lwoffii/wt offspring groups; shown are mean  $\pm$  SEM; n= 10 mice/study group)



**Fig.4.2.1b** Effect of prenatal exposure to non-pathogenic bacteria such as, Gram-negative bacteria (*Ac. lwoffii*) on airway inflammation in OVA - immunized and allergen challenged mice. Wild-type and TLR<sup>+/-</sup> offspring mice, C57BL/6 background were OVA -immunized. Both of the wt/ko *Ac. lwoffii*-exposed, respective wt/ko PBS-exposed groups were OVA aerosol challenged followed by a lavage of the airways. Cellular composition of BALF, such as: macrophages, neutrophils and lymphocytes are depicted (\* $P < 0.05$  and \*\* $P < 0.01$  *Ac. lwoffii*/wt versus PBS/wt; @ $P < 0.05$  *Ac. lwoffii*/wt versus *Ac. lwoffii*/k.o. offspring groups; § $P < 0.05$  PBS/k.o. versus PBS/wt. offspring groups; # $P < 0.05$  *Ac. lwoffii*/k.o. versus PBS/k.o. offspring groups; shown are mean  $\pm$  SEM;  $n = 10$  mice/study group)



**Fig.4.2.1c The effect of maternal exposure to *Ac.lwoffii* on offspring cytokine.** BAL fluids cytokines IL- 5, IL- 10, IFN- $\gamma$  levels from OVA-sensitized and challenged mice were measured by ELISA ( $^{\#}P < 0.05$  and  $^{##}P < 0.01$  *Ac.lwoffii*/k.o. versus PBS/k.o. offspring groups;  $^{§§}P < 0.01$  PBS/k.o. versus PBS/WT;  $^{@@@}P < 0.001$  *Ac. lwoffii*/wt versus *Ac. lwoffii*/k.o. ; shown are mean  $\pm$  SEM; n= 10 mice/study group)

**Fig.4.2.1d Effect of prenatal *Ac.lwoffii* exposure on airway-responsiveness to Methacholine.** Analysis done by Head-out-body plethysmography method. Both *Ac.lwoffii*-exposed wt/ko, respective wt/ko PBS-exposed groups were OVA-sensitized/-aerosol challenged followed lung function analysis ( $^*P < 0.05$  *Ac.lwoffii*/wt vs. ctrl; shown are mean  $\pm$  SEM; n= 10 mice/study group)

Moreover, lung function analysis was carried out using the non-invasive method of *head-out body plethysmography*. The middle-expiratory airflow (EF<sub>50</sub>) of bronchial responsiveness to  $\beta$ -Acetyl-Methacoline (MCh) was measured 24 hours after the last OVA aerosol challenge. Maternal Ac. lwoffii treatment reduces airway reactivity in wild type offspring. However, since there is no difference between WT/Ac. lwoffii and KO/Ac. lwoffii groups it cannot be state definitely if airway reactivity is affected through TLR signalling pathway.

#### **4.2.2 The effect of maternal non-pathogenic bacterial exposure on offspring spleen: cytokine and chemokine expression after in vitro OVA restimulation**

Cell counts of splenocytes (MNCs) and re-/stimulation with OVA, was done after 48 hours from the last allergen challenge. Total spleen cell counts were isolated by density gradient centrifugation (Lympholyte M, Cedarlane, Burlington, MA, USA). After three washing steps in PBS the mononuclear cells (MNCs) were counted by CASY TT cell counter system. Statistical representation of the total spleen cell counts in asthmatic WT offspring shown that are affected by acting through a different mechanism than TLR2, TLR3, TLR4, TLR7, TLR9. After counting, the splenic mononuclear cells were seeded into a in 24-well flat-bottom plates at 37°C in a cell incubator, under 5% CO<sub>2</sub> and 95% humidified atmosphere.  $2 \times 10^6$  cells/ml/well were stimulated with 50 µg/ml OVA in a total volume of 1 ml cell suspension. After 72 hours cell-free supernatants were harvested and levels of IL-4, IL-5, IL-10, IL-13, TNF $\alpha$ , IFN $\gamma$  were measured using Enzyme-linked immunosorbent assay (ELISA). Spleen supernatants measurement cytokines such as IFN $\gamma$ , IL-13, IL-4 and IL-5, no differences seen between wild type and knockout mice, un-/treated groups, but TNF $\alpha$  cytokine is reduced after Ac.lwoffii supplementation.

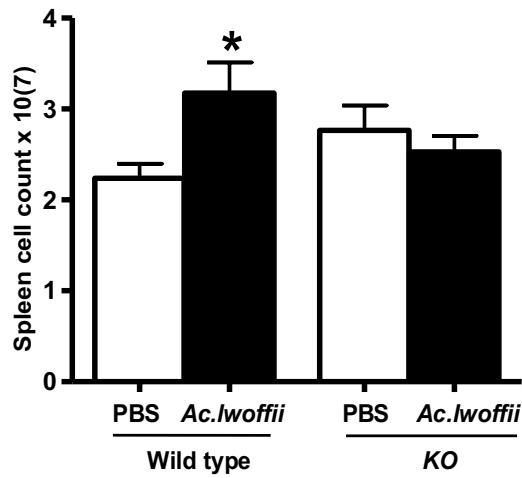


Fig.4.2.2a Total spleen cell counts after a prior isolation by density gradient centrifugation. (\*P < 0.05 *Ac.lwoffii*/wt vs. ctrl; shown are mean  $\pm$  SEM; n = 10 mice/study group)

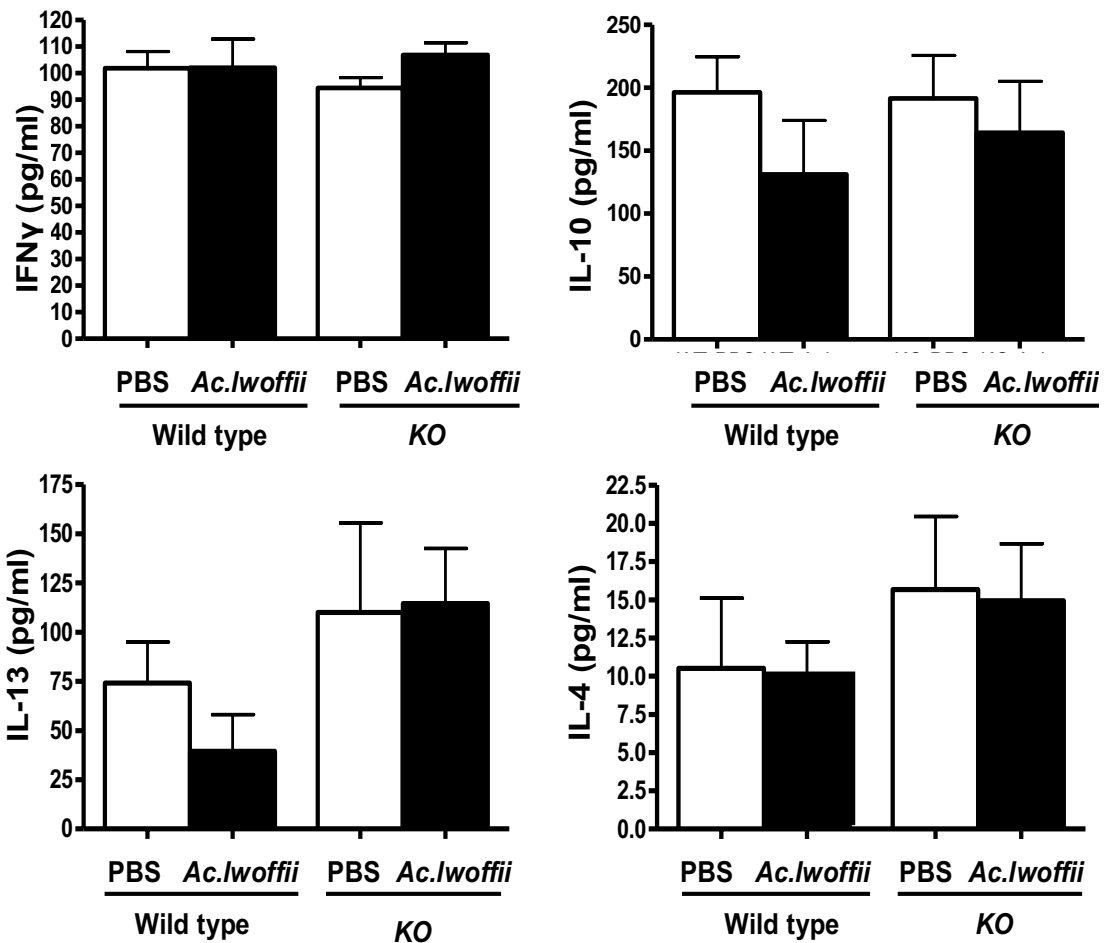
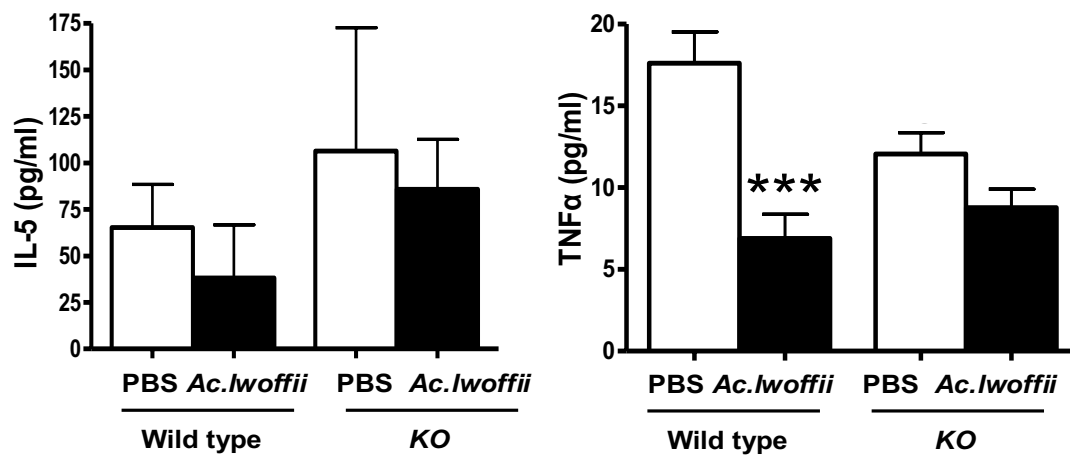


Fig.4.2.2b Measurement levels of IL-4, IL-10, IL-13 and IFN $\gamma$  using Enzyme-linked immunosorbent assay (ELISA), from 72h OVA restimulated cell-free supernatants. No significant expression of cytokine level in supplemented groups vs ctrl.



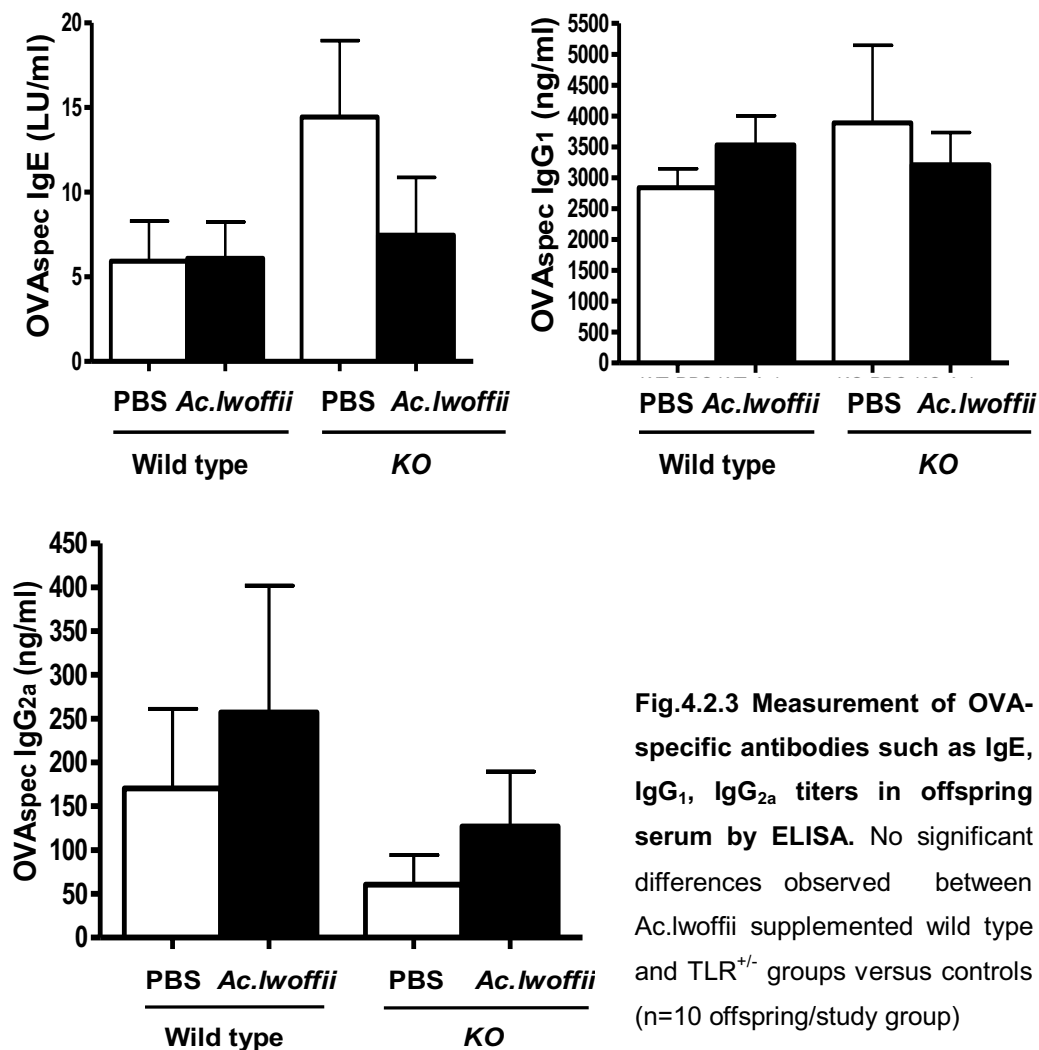


**Fig.4.2.2c Measurement levels of IL-5 and TNFα using Enzyme-linked immunosorbent assay (ELISA), from 72h OVA restimulated cell-free supernatants.** TNFα levels is significant reduced in of Ac.lwoffii/wt group in comparison with control(\*\*\*P< 0.001 Ac.lwoffii/wt vs. ctrl; shown are mean ± SEM; n= 10 mice/study group).

#### 4.2.3 The effect of maternal non-pathogenic bacterial exposure on offspring blood serum antibodies titres: OVA-specific IgG1, IgG2a and IgE measurements by ELISA

Offspring of prenatally Ac.lwoffii-exposed mother mice, strain C57BL/6 deficient mice, at age of 28 and 42 days were sensitize to OVA by two intra-peritoneal injections of 10 µg OVA (grade VI; Sigma, Deisenhofen, Germany) emulsified in 1.5 mg Al(OH)<sub>3</sub> (Pierce Co., Rockford, IL, USA) in a total volume of 200 µL. Later on, the animals received three local allergen challenges performed by an exposure to aerosolize OVA (1% in PBS) for 20 minutes on days 48, 49 and 50. Twenty-four hours after the last exposure to OVA the blood was collected and blood serum OVA/specific antibodies titers was performed by ELISA technique. Levels of OVA-specific IgG<sub>1</sub>, IgG<sub>2a</sub> and IgE antibody titers were measured by the ELISA technique. Briefly, 96 – well microtitre plates (Nunc, Wiesbaden, Germany) were coated with Ovalbumin (grade VI; Sigma GmbH, Germany) at a 4°C. After incubation, plates were washed (0.1% Tween 20/PBS) and blocked

with 1% bovine serum albumin (BSA)/PBS (w/v) for 2 hours at room temperature. Diluted serum samples and monoclonal OVA-specific IgE (Serotec, Düsseldorf, Germany), IgG<sub>2a</sub> (Dianova GmbH, Hamburg, Germany) and IgG<sub>1</sub> (Sigma) standards were added and the plates were incubated overnight at 4°C. After the plates were washed, biotin-conjugated anti-mouse IgE, IgG<sub>1</sub> or IgG<sub>2a</sub> (BD Biosciences Co., San Diego, U.S.A.) were added and incubated for 2 hours at room temperature. As a second-step reagent horse-radish peroxidase-conjugated streptavidin (Sigma GmbH, Germany) was added for 30 minutes (room temperature, devoid of light). Plates were developed with peroxidase substrate (Roche GmbH, Mannheim, Germany).

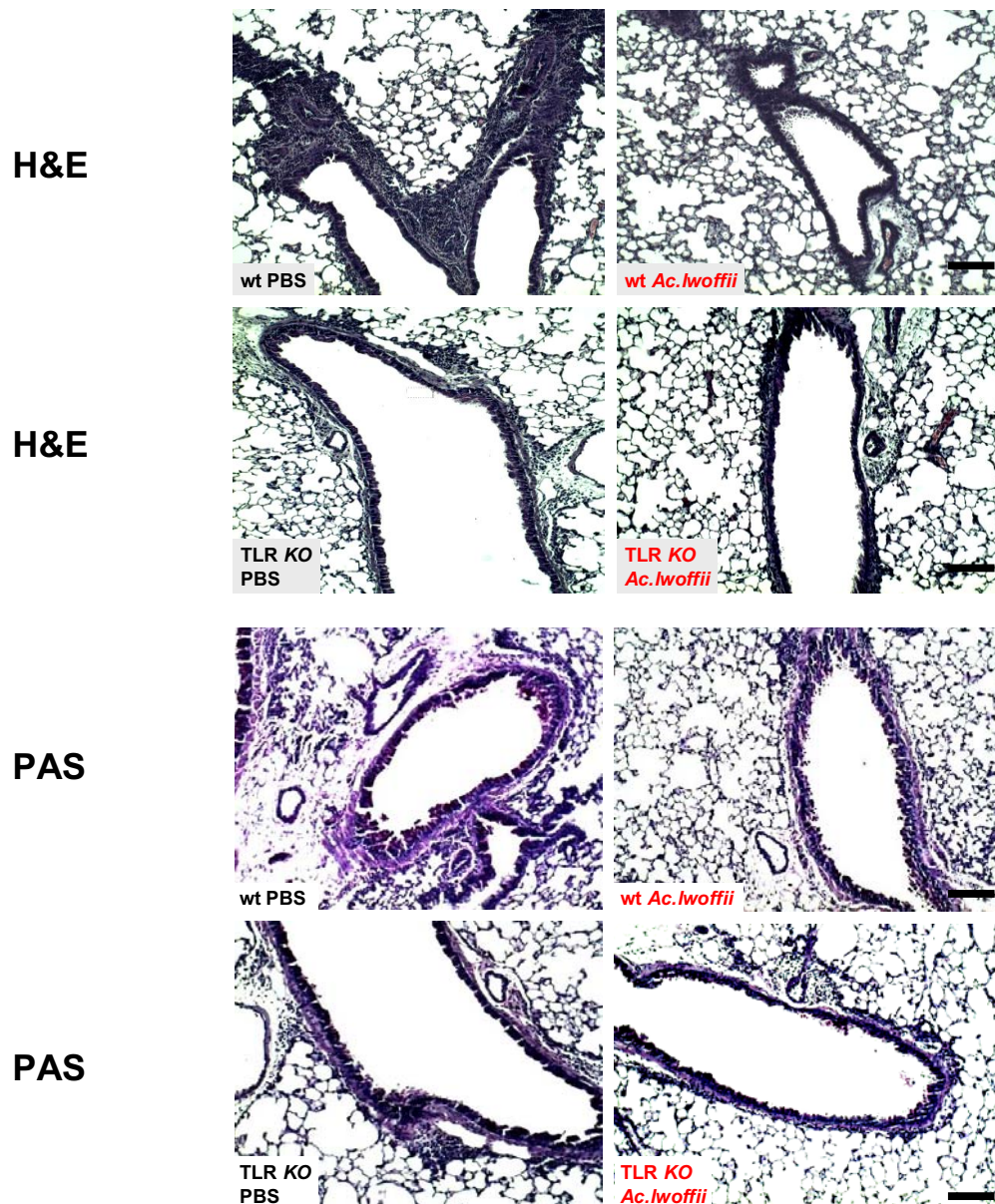


**Fig.4.2.3 Measurement of OVA-specific antibodies such as IgE, IgG<sub>1</sub>, IgG<sub>2a</sub> titers in offspring serum by ELISA.** No significant differences observed between *Ac.lwoffii* supplemented wild type and TLR<sup>+/-</sup> groups versus controls (n=10 offspring/study group)

Microtitre plates were read in an ELISA reader (Tecan GmbH, Crailsheim, Germany). Trends in IgE antibody show a reduction in KO/A.lwoffii in comparison with controls KO mice, but no significant differences seen in the antibodies of any of the groups.

#### **4.2.4 The effect of maternal non-pathogenic bacterial exposure on offspring lung: histopathological examination**

Histopathological examination of young mice lungs was done, after a prior perfusion with PBS buffer (pH 7.2) and fixation in situ with Formaldehyde 4% (wt/vol) sol. Tissue characterization and cell profiles of inflammatory cells were observed in Haematoxylin and Eosin (H&E), respective Periodic acid-Schiffs (PAS) stained sections, using High-Power Light Microscopy (HPLM). Lung histology revealed local reduction of the inflammatory cells and mucus secretion into wild type supplemented mice, but no changes seen in knockout mice in comparison with controls. To address the role of TLR signaling in prenatal asthma prevention, we designed an experimental system in which recognition of a broad spectrum of pathogen-associated-molecular-patterns (PAMPs) was selectively impaired in the mother. Since MyD88<sup>-/-</sup> and TNFp55<sup>-/-</sup> mice lack of TLR activity, respective IL-1 and TNF $\alpha$  cytokine family member signaling, involved in the regulation of inflammatory reaction, we considered these mice inappropriate for our study aim. In general, mice of the C57BL/6 background per se develop less airwayhyperreactivity (AHR). However, a significant improvement in lung function could be detected in TLR<sup>+/-</sup> sensitized offspring of TLR<sup>+/-</sup>/Ac.lwoffii exposed females, whereas TLR<sup>+/-</sup> offspring of TLR<sup>-/-</sup>/Ac.lwoffii females did not show any improvement. Therefore, we as other past studies demonstrated that in TLR-mediated signals are essentially to support allergy and asthma protection. Thus, asthma-induced experiment in TLR2/3/4/7/9<sup>+/-</sup> offspring required a total TLR signaling competence, finally resulting in the T<sub>H</sub>1 fetal developing immunity, particularly in asthma protective effects.



**Fig.4.2.4 Mice offspring lung histo-morphological illustration (WT/TLR<sup>+/-</sup> offspring, supplemented with PBS/Ac. Iwoffii) after OVA - sensitization/challenge.** Lung tissue cross-sections are stained either with haematoxylin-eosin (H&E, upper pictures) and periodic acid-Schiff (PAS, lower pictures). Haematoxylin stains cell nuclei blue, while eosin is strongly absorbed by red blood cells, colouring them bright red. Furthermore, it stains cytoplasm, connective tissue and other extracellular substances in pink or red. Periodic acid-Schiff staining is used to mark carbohydrates (glycogen, glycoprotein, proteoglycans) and to distinguish different types of glycogen storage diseases. Here, we monitor mucus producing goblet cells and airway inflammation in offspring. Scale bars represent 200  $\mu$ m.

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### 5.1 Introduction into research and methodological aspects

From methodological point of view, the present doctoral thesis addresses experimental science research dealing with the evaluation of experimental protocols and data in the area of Biomedical Science, particularly Immunologically Allergy. The doctorate's thesis theme: *The role of Toll-like receptors in the development of immunological tolerance in neonates* is part of the research project so-called *Sonderforschungsbereich* (SFB - TR22/A18) such as: *Mechanisms of pre- and postnatal instruction of allergic respiratory diseases in the mouse model of experimental asthma*. Furthermore, in the present research was possible to identify two important bacteria strains having the capacity to suppress and, respectively to prevent the development of experimental asthma. The bacteria strains: *Lactobacillus rhamnosus* GG (LGG, Gram<sup>+</sup> bacteria) and *Acinetobacter lwoffii* F78 (Ac. lwoffii F78, Gram<sup>-</sup> bacteria), have been originally identified in the epidemiologic longitudinal study, so-called PASTURE STUDY, which is an EU funded project under the leadership of the Technical University of Munich, Bayern, Germany. The relevant concepts are well defined, in addition to issues of research design and data gathering which are carefully discussed. Accordingly, the development of a mouse model of prenatal exposure to microbial compounds and the involvement of a molecular mechanistic design against allergic disorders, were imperiously required in this study. The offspring were analyzed with regard to the development of allergic phenotypes later in life. Thus, the first step was to acquire a broad knowledge in different read-outs including clinical investigations and secondly, it was necessary to set-up a number of assay systems, which allow us further analyzing the immuno-competence of placental tissue in mice (immunological medicine interference with cell- and molecular biology). The second step associates the asthmatic phenotype shown in offspring with the maternal protective molecular mechanism. The script confers the impasses as well as achievements gained throughout both, theoretical and experimental work. In an outlook on possible future developments, the aspects of methodology evaluation research and the

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acquired data prove that the instruction of infant immune responses in the perinatal period develops under the influence of maternal immunity.

## **5.2 Lactobacillus rhamnosus GG and Acinetobacter Iwoffii F78 bacteria strains – a perinatal regulation of allergic respiratory disease completed in mouse model of experimental asthma**

Our postnatal - acute asthma - established animal model used Balb/c and C57BL/6 mouse strains, perinatal exposed to bacterial allergens, such as LGG (Gram<sup>+</sup> bacteria strain, present in probiotic products), Ac. Iwoffii F78 (Gram<sup>-</sup> bacteria strain isolated from stable dust present on farmer mammalian skin) and Lipid A (the LPS's active component of E.coli a Gram<sup>-</sup> bacteria membrane). We observed that several mechanisms have been occurred in vitro (line-cell culture) and in vivo (animal studies) during the perinatal allergen exposure influencing the postnatal immunity but the related molecular- and cellular mechanisms with special emphasis on the prototypically Toll-like receptor ligands was more suggestive in the present work. Direct modulation of the immune system may be through the Toll-like receptor signaling pathways in concert with steroid hormones generating an anti-inflammatory cytokine cascade, increasing maternal production of IgG and secretory IgA, which eliminates antigens either from the mother and fetus intestinal mucosa and placenta. The idea is supported by the fact that allergic infants have been observed to have an aberrant intestinal microbiota. Clinical trials have shown that the standard treatment of infants with atopic diseases, can be significantly improved through the supplementation of LGG to pregnant women and subsequently, to their infants through the first months of extrauterine life and their exposure to farming environmental allergens in the perinatal stage.

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### 5.3 The essential responsibility of APC↔T cells cross-talk at foeto-maternal interface in shifting the infant immune system: a bias against T<sub>H</sub>1-regulated in concert to a cell-mediated immunity to diminish the progress of asthma in children at-risk

The APC ↔ T cell-mediated immune processes also play a fundamental role in the postnatal life. In order to fulfill their function as antigen presenters, APC serve two major functions: (i) they capture the proteins from pathogens or cellular debris and process them in that they digest them into fragments called epitopes, and (ii) APC present these epitopes on either class I or class II MHC proteins for T lymphocyte recognition. In addition to MHC presentation of peptides, APC express signals required for the proliferation and differentiation of T lymphocytes that specifically recognize the presented antigen. Maternal immunity is gradually suppressed during pregnancy from the beginning to the end similar as a foreign organ is transplanted, but here the fetal tissue expresses *histocompatible allergens* either from father and mother. The idea behind supported by prior studies was to characterise in our prenatal model the main immune cell classes (T, APCs, NKs) and some subclasses related to CD45<sup>+</sup> cells (regulatory T cells, activated T cells and activated T<sub>regs</sub>). They cross placenta during pregnancy and contribute in maintenance of the foeto-maternal immunity balance, within specific circumstances. We preferred to investigate the LA/LPS supplemented group, since postnatal data (Blümer, 2005) demonstrated that LPS in certain concentration are able to modulate allergic reactions. Here, we observed only a trend in induction of CD3<sup>+</sup>CD8<sup>+</sup> T cells, but a significant suppression of NK cells in LA supplemented group versus control. Furthermore, FACS analysis demonstrated a significant decrease in placental NK cells of the LA treated group versus controls. A high percentage of CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> fetal thymus cell subset or γ/δ T cells subset is present in term placenta in high percentage (44%). Alternatively, they are involved in cytokine-mediated immunoregulation of the maternal immune response producing T<sub>H</sub>2-/T<sub>H</sub>3-type cytokines, which result in

placental development, in local immunosuppression and immunomodulation (Santoni, 2006).

### **5.3.1 Alternative *face-book* of Asthma: the postnatal anti-allergic phenotype development is managed by cytokine expression at materno-fetal interface**

Furthermore, our observations including variation in  $T_H1/T_H2$  ( $T_H3$ ) cytokine production was considered either in stem cell trophoblasts and term trophoblasts. It must be a fine balance between cytokines with light induction into  $T_H2$  direction. The cytokine are promoted from both sides: placental trophoblasts both including stem trophoblasts and term trophoblasts as well and by decidual lymphocytes. A helping-hand in this regard is given by steroid hormones, which demonstrated a consistent contribution inducing the expression of placental cytokines from implantation phase to term stage. Also, trends in supplemented mother mice include an increase in placental  $T_H2$  cytokine such as IL-4 and IL-10 in LGG and LA, whilst in Ac. Iwoffii group the production of these cytokines were inhibited. It might be that LA, the active component of LPS is metabolized during systemically absorption, respectively in the lipid metabolism. Consequential appear dissimilarities into LA and Ac. Iwoffii experimental results. Proinflammatory cytokine like IL-1 $\beta$  was upregulated in LGG and Ac. Iwoffii mother groups, whereas in LA mothers the effect is opposite. Also, significant differences between all supplemented groups include an inverse relationship between IFN $\gamma$  and TNF $\alpha$ . Certain adaptor molecules, mediators like cytokine and chemokine, and transcription factors involved in TLR signaling pathways at mRNA level (NF-kB, MCP-1, T-bet, GATA-3, FoxP3, CD3, CD14, CD11), playing a key role in regulation of inflammatory response were also evaluated. We remarked only an upregulation of MCP-1 expression in supplemented mothers in comparison with controls. The expectation is achieved since the chemokine is involved in the complement system annihilating foreign pathogens. The monocyte chemotactic protein-1 (MCP-1) or CCL2 recruits monocytes, memory T cells, and dendritic cells to sites of allergen reaching tissue. CCL2 causes the

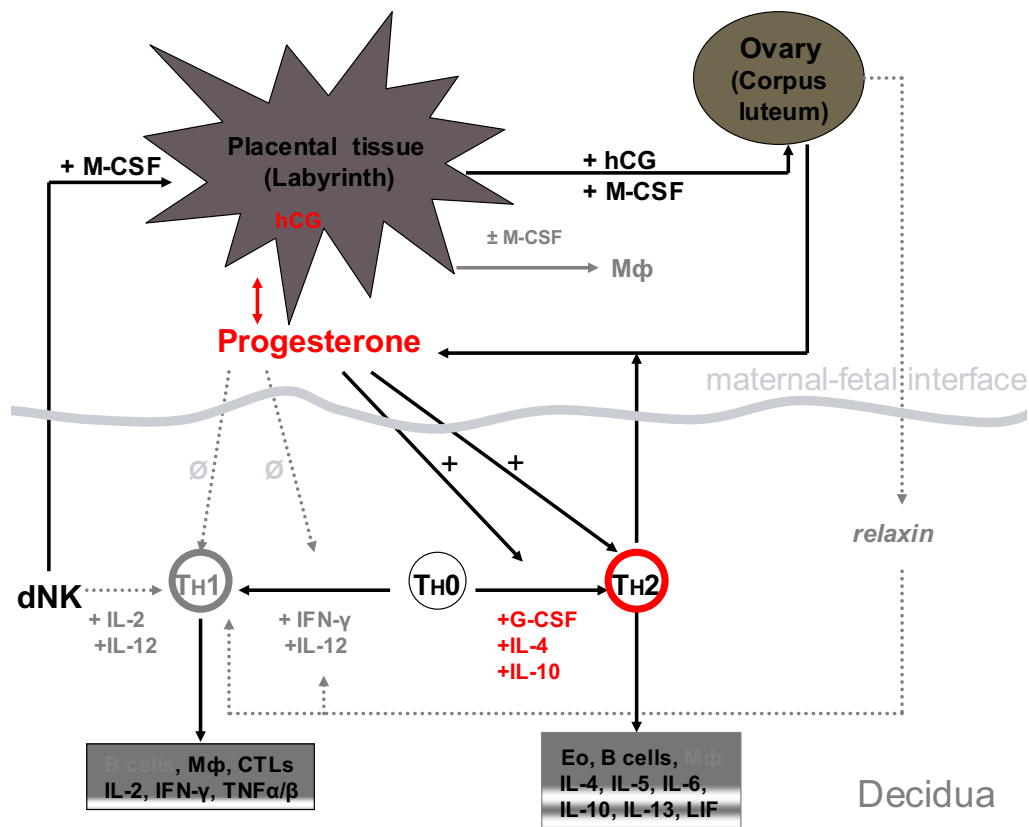


degranulation of basophils and mast cells. Also, mRNA CD3 expression illustrates an upregulation at mRNA level indicating the presence of T<sub>H</sub>2 cell in decidual tissue. Besides, in stem trophoblasts the mRNA T<sub>H</sub>1/T<sub>H</sub>2 cytokine expression diverge from stimulated cells to another. IL-1 $\beta$  and IL-4 expression is upregulated in stimulated cells versus controls. Moreover, IL-4 a T<sub>H</sub>2 cytokine is present either in stem- and term trophoblasts as well mediating the activation, differentiation and dedifferentiation of B cells, promoting class switch IgG<sub>1</sub> and IgE important in immunity transfer from mother to the fetus. Trends include an inverse relationship in stimulated placental stem cells in T<sub>H</sub>2 cytokines such as IL-10 at mRNA level, while IFN $\gamma$  and TNF $\alpha$ , are not expressed at all. Comparable, these two experiments done with stem- and term trophoblasts quantifying the mRNA T<sub>H</sub>1 and T<sub>H</sub>2 cytokine demonstrated their promising functions in pregnancy and influence of fetal immunity. The proinflammatory cytokine IL-1 $\beta$  induced in both type of cells play an important role in implantation when the morphology of the uterus changed becoming amplified when the pregnancy achieves its term. IL-1 $\beta$ , similar to TNF $\alpha$  attracts leucocytes and endothelial adhesion molecules at inflammation site inducing acute-phase proteins by activating lymphocyte co-stimulation phagocyte. Also, it promotes the prostaglandin synthesis and tissue remodeling avoiding any pregnancy pathogenesis.

### **5.3.2 Steroid hormones: a *bridge-like* between endocrine and immune systems and their regulatory role upon pregnancy T<sub>H</sub>1/T<sub>H</sub>2 (T<sub>H</sub>3) cytokine balance**

Progesterone and Estradiol from supplemented mother mice blood serum and amniotic fluid were quantified by ELISA and CBA kit. The experimental protocol has been established for five different groups of mouse females: (i) Nonpregnant /Nonsupplemented (blood serum), (ii) Pregnant/Nonsupplemented used as Control for all supplemented groups , (iii) Pregnant/PBS (control group), (iv) Pregnant/LGG, (v) Pregnant/Lipid A, (vi) Pregnant/Ac. Iwoffii supplemented groups. Our observations reveal that in serum the progesterone level shown a

significantly up-regulation only in the Ac. Iwoffii supplemented females, while the estradiol level is suppressed in LA supplemented group, related to control group. The hormonal variation given by pregnancy state is associated with bacterial supplementation and persuades fetal immunity. Furthermore, lacking of classical MHC class -I and -II antigens at the trophoblast sides induces immunological processes under hormonal control, preserving the  $T_H1/T_H2$  cytokine balance. In contrast to the peripheral blood, at the feto-maternal interface there is present a certain composition of immune competent cells. Also, many different interactions between the endocrine and immunological system occur, where the steroids hormones modulate the secretion of certain cytokine and vice-versa. Similar measurements are performed either in blood serum and amniotic fluid of important cytokine, such as IL-4, IL-5, IL-10, IL-12p70,  $TNF\alpha$  and  $IFN\gamma$ . The results reveal no significant difference between bacterial supplemented and control groups. Nevertheless, it is expected that relatively a slight perturbations of the  $T_H1/T_H2$  balance can have an impact, either decreasing or increasing a risk of later allergic and/or airway diseases. As discussed, cytokine and chemokine play a very important role in the reproduction (i.e. embryo implantation, endometrial development, and trophoblast growth and differentiation) by modulating the immune and endocrine systems. The close correlation between the embryo and endometrium, and between the placenta's labyrinth and decidua are mediated by sex steroid hormones, cytokine and chemokine. As a result of this closely related cross-talk, pregnancy is successfully maintained. Production of cytokine including interferon-gamma ( $IFN\gamma$ ) and tumor necrosis factor-alpha ( $TNF\alpha$ ) is an important early-stage host response following infection with intracellular pathogens. Numerous studies have shown that early cytokine responses involving IL-12 and  $IFN\gamma$  are important for resistance to intracellular pathogens, whereas responses involving IL-4 and IL-10 increase host susceptibility. The early cytokine responses control the differentiation of naïve  $CD4^+$ T helper cells into  $T_H1$ - and  $T_H2$ -type cells.



**Fig.5.3.2 Progesterone-mediated interaction between endocrine and immune system-** Progesterone promotes the differentiation of  $T_H2$  cells and the release of  $T_H2$ -cytokine (IL-4, IL-5, IL-6, IL-10, IL-13), inhibiting the  $T_H1$ -cells differentiation and production of  $T_H1$ -cytokine. Additionally, progesterone increased the concentration of macrophage-cell stimulating factor (M-CSF), necessary for differentiation and proliferation of the trophoblasts, controlling the macrophage functions. Also, M-CSF is produced by dNK cells and promotes the secretion of human Choriogonadotropin (hCG) hormone by trophoblasts, which activates corpus luteum in production of progesterone and relaxin. Relaxin and progesterone are antagonistic of the  $T_H1/T_H2$  balance. Relaxin promotes the differentiation of  $T_H0$  into  $T_H1$  cells and the secretion of  $T_H1$ -cytokines (IL-2, IFN $\gamma$ , TNF $\alpha/\beta$ ). IFN- $\gamma$  is secreted also of dNK-cells in presence of IL-12 and IL-2. The differentiation of  $T_H0$  cells in  $T_H2$  cells is determined by granulocyte-cell stimulating factor (G-CSF), IL-4 and IL-10, and in  $T_H1$ -cells by IFN $\gamma$  and IL-12.  $T_H2$ -cytokines promote the functions of eosinophils and B cells and inhibit several macrophage functions. Thus, they support the cell-independent humoral immune response. The  $T_H1$  cytokine production activates macrophages, funded CTLs (cytotoxic T lymphocytes) and inhibits B-cells, as an effect of the phagocytosis-mediated cellular immune response (according to: Geis K., Dietl J., Die Rolle der Zytokine an de fetomaternalen Grenze, 2001. Zentralbl Gynakol, 123:609-618)

The characterization of CD4<sup>+</sup>T helper cell responses as T<sub>H</sub>1 or T<sub>H</sub>2-type is based mostly on the cytokine profiles and has been used in recent years to account for the innate resistance and susceptibility of different inbred strains of mice to several intracellular pathogens (Ulett, 2000). The apparent paradox that embryo is not rejected by the maternal immune system despite the presence of paternal MHC histocompatibility antigens has been explained in mice by a T<sub>H</sub>2 switch at the level of the materno-fetal interface. Therefore, steroid hormones enhanced during pregnancy can affect the development of CD4<sup>+</sup>T helper cells into type-1 (T<sub>H</sub>1) or type-2 (T<sub>H</sub>2) effectors and responses, respectively. *Progesterone*, at concentrations comparable to those found at foeto-maternal interface, promotes the production of IL-4 and IL-5, whereas *relaxin* promotes the production of IFN $\gamma$  by T cells. Additionally, other factors such as leukaemia inhibitory factor (LIF) essential for embryo implantation associates with T<sub>H</sub>2 cells and is up-regulated by IL-4 and progesterone. Thus, LIF is down-regulated by T<sub>H</sub>1 inducers (IL-12, IFN $\gamma$  and IFN $\alpha$ ). Furthermore, a decreased production of LIF, IL-4 and IL-10 by decidual T-cells was remarked from many studies, in women with recurrent abortions in comparison with women with normal gestation. But the decreased production of LIF, IL-4 and IL-10 was not observed in peripheral-blood T-cells. These results suggest that the local production of LIF and/or T<sub>H</sub>2 cytokines may contribute to the maintenance of pregnancy. Furthermore, T<sub>H</sub>1-type cytokines promote allograft rejection compromising pregnancy, whereas T<sub>H</sub>2-type cytokines inhibit T<sub>H</sub>1 responses and allow allograft tolerance. Thus, a direct cause↔effect relationship between the defective production of LIF, macrophage-stimulating factor (M-CSF) and T<sub>H</sub>2-type cytokines produced by T cells present at foeto-maternal interface and the pregnancy loss has been observed (Piccinni, 2000).

### **5.3.3 TLR expression in maternal tissues is imperiously required in prevention of allergic diseases**

While the clinical benefits of prenatal and early postnatal LGG consumption are limited, but clearly indicates the demand using microbes or microbial components new opportunities for further exploration. Recent, experimental animal models

(mouse) for various allergic phenotypes including acute experimental asthma with the development of airway remodelling was designed in our laboratory. We observed that intestinal colonization with LGG as observed in mother mice only, but not in the offspring (Blümer, 2007). Furthermore, in LGG maternal supplemented small intestine, the mRNA TLR4 expression shows a high induction in the 1<sup>st</sup> trimester of gestation but not mRNA TLR2 which is insignificant in comparison with control group. Also, in the 3<sup>rd</sup> trimester of pregnancy the expression of mRNA TLR2 and TLR4 from LGG mothers remain insignificant in association with control group. Consequently, bacterial DNA is highly enriched in immunomodulatory DNA containing CpG motifs that bias the immune response to antigens and thus, influence and developing an anti-allergic immune response in infancy. Moreover, maternal LGG supplemented spleen mRNA TLR4 expression shown an induction only in 1<sup>st</sup> trimester of pregnancy but not mRNA TLR2, while in the 3<sup>rd</sup> trimester the expression at spleen mRNA level of both TLR is more equilibrate in supplemented group in association with control group. In conclusion, the first maternal immunity suppression signal during pregnancy which appeared in immune organs as small intestine and spleen is represent by increasing of TLR4. The immunosuppression is balanced with evolution of gestation, being influenced by increasing levels of other immunomodulatory factors such as cytokine and chemokine, antibodies and steroid hormones. The relevance for a potential therapeutically strategy against allergic asthma might met the *Acinetobacter lwoffii* F78 (Gram<sup>-</sup> bacteria), which is present in high amount into cowshed microflora. Besides, a variation in all ten placental mRNA TLR expression was remarked in supplemented groups in comparison with controls. We demonstrated that trends in LGG and LA supplemented mother mice embrace increasing of TLR1 and TLR9 and a dramatically suppression of TLR7, respectively. In *Ac. lwoffii* group mRNA TLR7 expression is upregulated, whilst TLR6 and TLR9 signals are suppressed. Differences in expression of TLR signals in supplemented LA and *Ac. lwoffii* groups include a high regulation in TLR4 and surprising in TLR5 at mRNA level, opposing to LGG supplemented mothers which showed an inhibition of TLR4

signal in comparison with controls. Steroid hormones, adaptor molecules and transcription factors, bacterial CpG could complete the picture of TLRs signaling dissimilarity in placental tissue at term. Additionally, in vitro bacterial stimulated Trophoblast Stem Cells do not shown any significance in mRNA TLR expression, with exception of those stimulated with Ac. Iwoffi mRNA TLR1 was upregulated after 24 hours. Thus, these comparative experiments in trophoblasts stem cells and dedifferentiated trophoblasts existing in term placenta, revealed the incapacity of immature placental stem cells at the uncommitted stage to express any TLR. The TSCs need the attendance of growing and dedifferentiation factors, which are normally present in vivo, such as cytokine and chemokine, thus being able to develop themselves and to complete their multifunction in maintenance the incomprehensible mechanism of tolerance and immunoprotection of pregnancy. It is thought that an adverse intrauterine environment during a critical stage of development permanently alters or “programs” the development of fetal tissues, by influencing the gene expression and this allows the fetus to survive but with critical consequences in postnatal life. Nevertheless, the expression of placental TLR by IHC or IHF was not properly elucidated since a specific antigen-retrieval and specific placental enzymatic inhibitors were not commercial available.

#### **5.3.4 Farming allergens: the best *educators* for the prenatal epigenetic programming of the postnatal T<sub>H</sub>1 immunity**

In early mammalian development (fertilization to eight-cell stage), the genome is demethylated. From the eight-cell stage to the morula, *de novo* methylation of the genome occurs, modifying and adding epigenetic information to the genome. By blastula stage, the methylation is complete. This process is referred to as *epigenetic reprogramming* (Mann, 2002; Weaver, 2007). The importance of methylation was shown in knockout mutants without DNA-methyltransferase which died at the morula stage. Increasing evidence is revealing a role of methylation in the interaction of environmental factors with genetic expression. Differences in maternal care during the first 6 days of life in the rodents induce

differential methylation patterns in some promoter regions and thus influencing gene expression (Nakayama, 2001). Furthermore, even-more-dynamic processes such as interleukin signaling have been shown to be regulated by methylation (Bird, 2003). Latest epigenetic studies indicate that some epigenetic mechanisms play a crucial role in the prevention or inception of chronic diseases, regulating the expression of specific genes by adaptive processes in the genome, either by *disabling* (transcriptional silencing) or by *activation* of genes without sacrificing the primary DNA sequence. The genomic adaptation processes are taught by environmental influences and modern lifestyle (synthetic and natural allergens). The potential perinatal mechanisms by which epigenetic processes in the silencing or activation of specific genes involved are currently being discussed: the direct chemical modification of the DNA region by methylation or by modification of proteins (histones)-closely associated with the corresponding locus and manipulates the accessibility of chromatin for the transcription (Grewal, 2004; Walsh, 2006). Furthermore, newer study shows that the diet (food allergens), particularly in the early development phase of the prenatal period, the stable gene expression in adulthood is already compromised (metabolic programming). Besides, we believe that Gram<sup>-</sup> bacterial membrane component LPS and bacterial DNA are the principal promoters in activation of the maternal immunoprotective mechanisms using TLRs pathways. It is well known that the bacterial DNA is highly enriched in immunostimulatory DNA containing CpG motifs that bias the immune response to antigens to a non-allergic T<sub>H</sub>1 phenotype. This property of bacterial DNA provides a potential mechanism for gastrointestinal bacterial DNA to modulate the developing immune response in infancy. Bacterial CpG motifs are a 6-base pair sequence of noncoding DNA, unmethylated and functional, whereas vertebrate CpG is methylated and lacks significant immunostimulatory activity. The CpG motifs act indirectly on T lymphocytes as they are taken up by cells of the innate immune system that secrete cytokines (IL-12, IFN $\gamma$ ), which bias naïve T lymphocytes to develop into T<sub>H</sub>1 cells. In mouse models of asthma, immunostimulatory DNA sequences inhibit eosinophilic inflammation, T<sub>H</sub>2 cytokines including IL-5, and

airway hyperreactivity to methacholine. The immunostimulatory DNA sequences are effective when administered systemically or mucosally. Studies in humans are currently evaluating whether the use of these immunostimulatory DNA sequences can inhibit allergic responses in humans as they do in animal models of allergy. Nowadays, it is known that an allergic individual is more sensitive than non-allergic ones to the bronchoconstrictive properties of inhaled LPS. An explanation for the directed bronchoconstrictor response to high doses of inhaled LPS is suggested from genetic studies demonstrating a mutation in the extracellular domain of the receptor that binds LPS, the toll like-receptor 4 (TLR4). The TLR4 receptor polymorphism is significantly associated with those individuals who are resistant to the bronchoconstrictor effects to high doses of inhaled LPS. But inflammation is a complex response to infection and tissue injury (Nathan, 2002), therefore we anticipated that Toll-like receptors (TLRs) have a crucial role in the inflammatory response to infection and allergy, especially in children through an immunity still vulnerable. Thus, bacterial lipopolysaccharide (LPS) signals through TLR4, being one of the most potent inducer of inflammation (Takeda, 2003). Multiple regulatory mechanisms could take place, controlling the degree and/or duration of TLR-induced inflammation: (i) the inhibition of TLR signaling by inducible negative regulators, (ii) production of anti-inflammatory cytokines and, (iii) alterations of the TLR signaling cascade (Liew, 2005). Collectively, these mechanisms contribute to the phenomenon of LPS tolerance: the transient unresponsiveness of cells or organisms to repeated or prolonged stimulation with LPS (Beeson, 1947a/b; West, 2002; Cavaillon, 2006). LPS tolerance has traditionally been viewed as a *hyporesponsive state* of macrophages resulting from receptor desensitization (Dobrovolskaia, 2002/2003; Fujihara, 2000/2003; Medvedev, 2000/2002). However, TLRs induce expression of hundreds of genes with different functions (Huang, 2001) and therefore different regulatory requirements. Thus, it is not known that all TLR-induced genes are controlled exclusively at the signaling level. This would not discriminate between gene subsets with distinct functions because not all TLR-induced genes have the potential to cause tissue damage. These include genes



encoding anti-microbial effectors that are essential for the early-host defense from infection. Thus, a transient disruption of TLR-induced expression of these genes would leave the host immunocompromised.

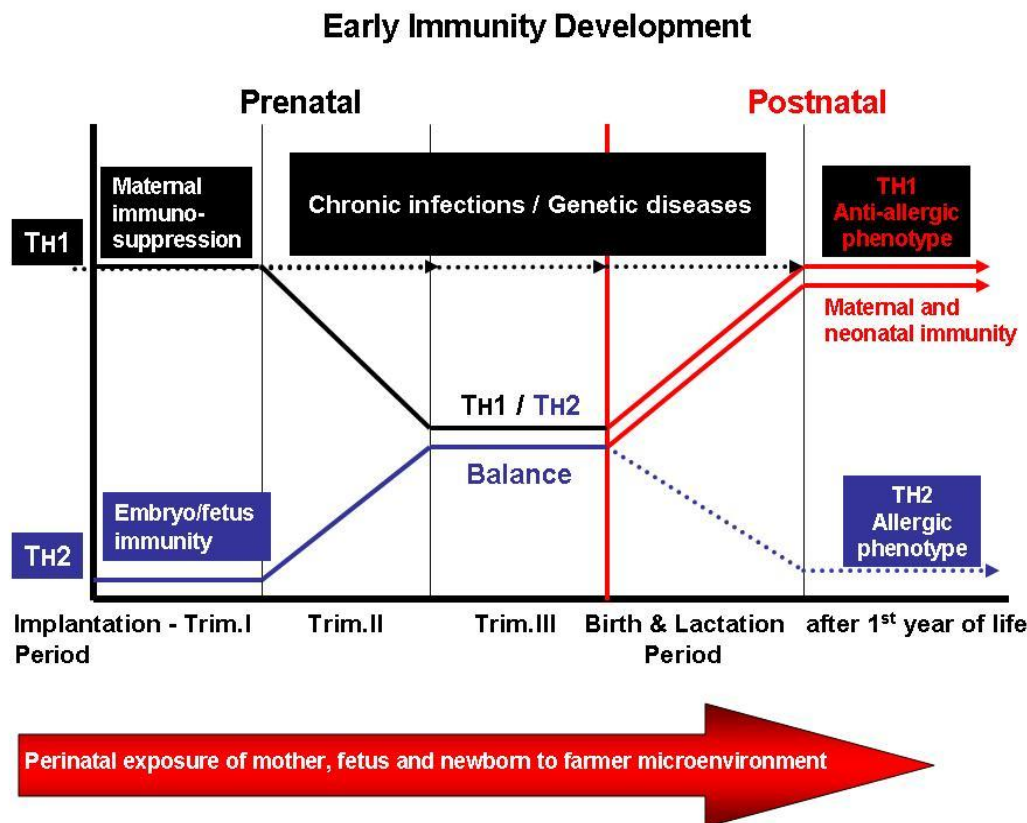
#### 5.4 Beneficial effects observed in mouse offspring prior perinatal farming allergens exposure

Since the innate immune system plays an essential role in the initial immune activation and modulates subsequent adaptive immune responses, we investigated in maternal tissues due to pregnancy period, where the existing *pattern-recognition receptors* (PRRs) such as *Toll-like receptors* (TLR) are involved through specific signalling pathways, after bacterial supplementation. Consequently, the postnatal experimental part corroborates mostly with the prenatal data and reveals together a potential protective molecular mechanism against allergies, such as asthma associated. Therefore, in this supplementary experimental protocol, providing that the TLR-mediate asthma defensive molecular mechanism, were used C57BL/6J strain female mice, 6-8 weeks old homozygote MyD88<sup>-/-</sup>, TLR<sup>-/-</sup> (TLR2/3/4/7/9<sup>-/-</sup>), TNFp55<sup>-/-</sup> versus wild type female mice (wild type male bred). In heterozygote asthmatic induced offspring we observed that *Acinetobacter lwoffii* F78 supplementation appears to act through maternal TLR, to reduce BAL cell - eosinophil and lymphocyte, and decreased number of macrophages. Furthermore, offspring of TLR knockout mice have decreased numbers of BAL macrophages. Moreover, *Acinetobacter lwoffii* action appears to be through maternal Toll-like receptors signalling pathway, but it thought that an alternative system occur to affect BAL neutrophils. To observe the effects of maternal exposure to *Ac. lwoffii* on offspring cytokine and more, the course of immune response, BAL fluids cytokines such as IL-10, IL-5, IFN $\gamma$  levels from OVA-sensitized and challenged mice were measured by ELISA method. Accordingly, in the absence of maternal TLR, *Ac. lwoffii* supplementation has an effect on asthmatic offspring BAL - IFN $\gamma$  levels. We observed a strong down-regulation of IFN $\gamma$  in KO/*Ac. lwoffii* supplemented group in relationship with

KO/PBS and WT/Ac. Iwoffii and WT/PBS groups. This result reveals an opposite effect, respectively an induction of  $T_H2$  immunity in comparison with the phenotype of asthmatic offspring Balb/c strain, which reveals a  $T_H1$  immunity – an up-regulation of BAL  $IFN\gamma$ . Also,  $TLR^{+/-}$  offspring cover reduced BAL IL-10 production, but no difference seen in BAL IL-5 levels. Moreover, lung function analysis was carried out using the non-invasive method of head-out body plethysmography. The middle-expiratory airflow ( $EF_{50}$ ) of bronchial responsiveness to  $\beta$ -Acetyl-Methacoline (MCh) was measured 24 hours after the last OVA aerosol challenge. Maternal Ac. Iwoffii supplementation reduces airway reactivity in wild type (WT) offspring. However, since there is no difference between WT/Ac. Iwoffii and K.O./Ac. Iwoffii groups it cannot be stated definitely if airway reactivity is affected through TLR signalling pathway. Therefore, the reduction of hyper-sensitivity reaction in OVA-sensitized offspring against controls littermates depends by the transferred maternal allergen-specific IgG<sub>1</sub> antibodies. This shows that unlike the offspring of control mice, the offspring of OVA-sensitized mice developed a qualitatively and quantitatively higher anti-allergen-specific immune response. An allergen re-exposure of these animals reveals a suppression of the allergen-specific IgE and IgG<sub>1</sub> production, even these animals could develop immediately a hypersensitivity reaction. These data demonstrate that the postnatal  $T_H2$  immunity suppression could be dependent on the perinatal transfer of the maternal antibodies IgG<sub>1</sub> subclass. Thus, this might be an explanation why some mouse strains are immunologically resistant by generating of an allergic phenotype (e.g. BL57C/6 background). In conclusion, all these studies prove evidence that the maternal immunity exercises a decisive influence on development of the adaptive immune response in the offspring. This character finds possible already prenatally, and operates in the early postnatal period. The basement of this character is the induction and instruction of a specific T-cell response in the child, educated by maternal adaptive- and innate immunity through IgG and TLR.

### **5.5 The immunity transfer from mother to child - a potential maternal anti-allergic immunoprotective mechanism evolved through pregnancy with effectiveness in spring age children**

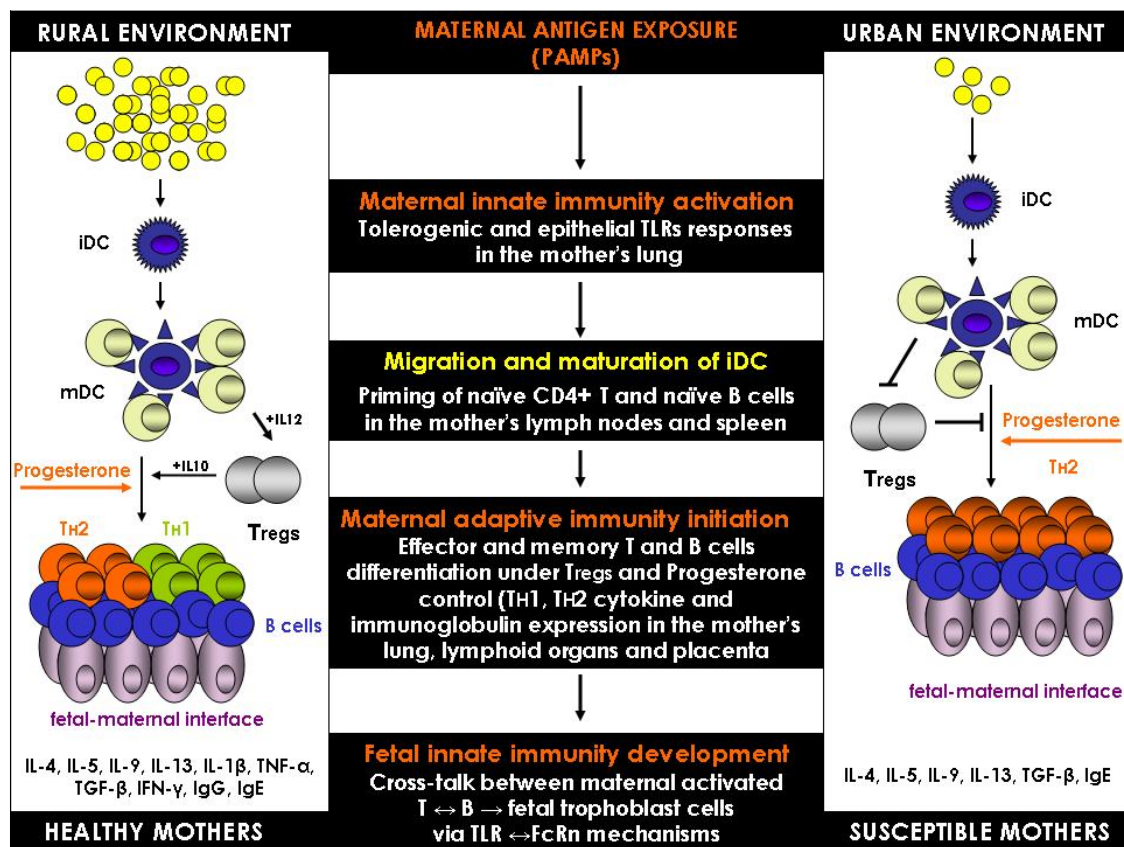
Atopic children are born in a healthy state. The earliest clinical manifestation of the atopic syndrome manifests not before the first few months of extrauterine life, and primarily location occurs on the skin tissue (atopic eczema), gut (food allergy) and later, into respiratory tract (asthma). In our focus, the direction of the promising process *per-allergic* vs. *anti-allergic* within the perinatal stage is essentially determined by two aspects: (i) suppression of maternal immunity during pregnancy (prenatally events - the maternal  $T_H1$  immunity is suppressed by intervention of paternal genetics); (ii) permanent exposure to micro-environment (allergens) where bacteria components operate like anti-allergic adjuvants *via* TLR pathways (postnatally events - the infant immunity switch from  $T_H2$  phenotype shortly present at birth into  $T_H1$ -type immunity during 1<sup>st</sup> year of life, developing an anti-allergic phenotype). Regarding, the particular importance of  $T_H1$ ,  $T_H2$  and  $T_{reg}$  cells in perinatal stage was monitored in the maternal tissues in concert with offspring's lung analysis. This complex molecular mechanism, the induction and instruction of a specific T-cell response (e.g. CD4) in the child might be mediated by innate regulatory pathways, such as Toll-like receptors jointly maternal humoral immunity. There is a correlation among maternal activated adaptive and innate systems through the environmental allergens which modulate the plasticity of the child immune responses. The transfer of antibodies is a mandatory molecular mechanism which occurs only in pregnancy and confers a compensated molecular protective mechanism: (i) prenatal via placenta from maternal bloodstream to fetal bloodstream; (ii) postnatal via colostrum and breast milk direct into baby gastrointestinal tract. The maternal antibodies offer a passive protection and supplementary, they are actively involved in development of the infant immunity. Also, maternal immunomodulatory molecules such as soluble factors and transfer factors play a key role in immunoprotective mechanism.



**Fig.5.5a Graphical illustration of the potential anti-allergic mechanisms developed during and after pregnancy stage and current deviations from a  $T_H2$  phenotype pregnancy.** Farmer non-pathogenic bacteria (bacterial antigens) could be responsible for baby's immunity switching from  $T_H2$  (before and shortly after birth, correlated with pregnancy well development) into  $T_H1$  (correlated with anti-allergic neonate phenotype). This mechanistic molecular machinery works only if the expecting mother and newborn - within the 1<sup>st</sup> year of life - are daily exposed to farmer environment.

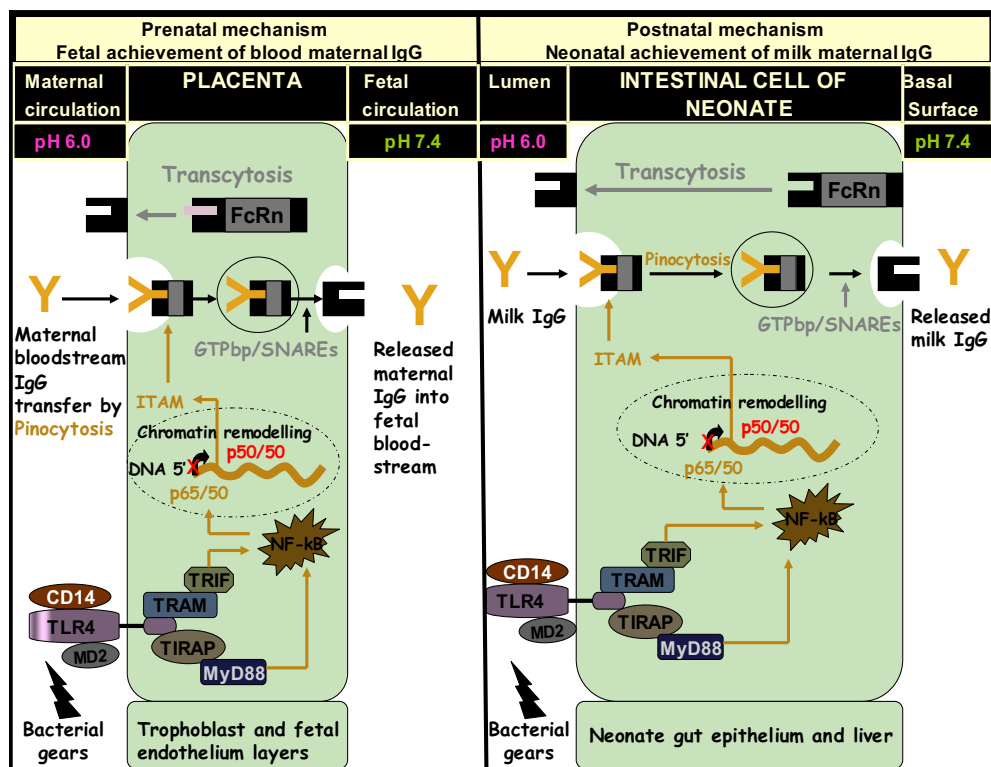
The important meaning of the fetal acceptance and immunoprotection desired effect of the pregnancy  $T_H2/T_H3$  immuno-deviation is the suppression of maternal  $T_H1$  immune responses by up-regulating T-cell functions at the maternal-fetal interface. Infections or environmental challenge may drive the maternal immune system towards a cell-mediated, like tolerogenic type response. In functioning immune responses, the helper- and cytotoxic T cells are activated by presentation of antigens to *antigen-presenting cells* (APCs). Following inhalation of pulmonary allergens, maternal lung antigen presenting cells (APCs) swallow,

the microbe begin to process antigen and migrate to peripheral lymphoid tissues, gut lymphoid- and placental tissue during pregnancy. In the maternal lymph node the APC and T cells link-up relative shortly after allergenic exposure in concert with paternal antigens, therefore the microenvironment of the lymph node significantly influences the developing T cell response in  $T_H1$  or  $T_H2$  direction. A shift in the maternal  $T_H1$  to  $T_H2$  immunity can result in pregnancy, as well as in allergic diseases. In mother, the innate response destroys or limits the pathological actions of allergens until adaptive immunity develops and then, the new concept is recognized. Any alteration in the maternal innate immune response can result in a radical fetal immune deviation.



The type of fetal immune response mounted during a prenatal microbial exposure is determined by the host innate response, genetics or microbial pathogenesis. A combination of these components determines the development of child  $T_H1$  or  $T_H2$  response which is dependent on the type cells and early signals from his innate immune system. The cells and signals of innate immunity provide the major immunoregulatory functions that link both innate- and adaptive immunity, which develops later during 1<sup>st</sup> year of extrauterine life. Besides, some maternal antibodies against fetal antigens are removed as immune complexes. It is well known that in primates, prenatal transfer of IgG from mother to offspring occurs predominantly across the placenta. Although in human placental tissue a number of Fc-receptors and IgG binding proteins, their involvement in IgG transport across the placental syncytiotrophoblasts have been detected. Therefore, the Fc receptor architectonic  $\gamma$ -mediated protective mechanisms appear as a top candidate molecule to explain the postnatal immunosuppression, in concert with maternal IgG<sub>1</sub> antibodies subclass. Fc receptors bind to antibodies that are attached to infected cells or invading pathogens. Their activity stimulates phagocytic or cytotoxic cells to destroy microbes, or infected cells by antibody-mediated phagocytosis or antibody-dependent cell-mediated cytotoxicity. The signal activates an inhibitory intracellular tyrosine-dependent-activation-motif (ITAM). The signal transduction cross-talk mechanisms by which ITAM-associated receptors modulates signaling through Toll-like receptors (TLRs), TNFR family members and cytokine receptors that use the JAK–STAT signaling pathway (Ivashkiv, 2009). ITAM-mediated cross-regulation can either enhance or diminish signaling by other receptors. Three major downstream effector signalling pathways are activated by ITAM-coupled receptors: NF- $\kappa$ B, MAPKs and calcium pathways. Of these effector signalling pathways, NF- $\kappa$ B and MAPKs are involved in cell activation and can also be activated by many other receptors, such as TLRs, and proinflammatory cytokine receptors, like the interleukin-1 receptor (IL-1R) and tumor necrosis factor receptor (TNFR). The high affinity IgG transporters are the larger protein the  $\alpha$ -chain of human FcRn (hFcRn) of ~45-kDa and a smaller  $\beta$ 2-microglobulin of ~15-kDa molecular weight.

FcRn binds IgG on the cell surface (intestine epithelium) or in endosome (trophoblast). Transcytosis by FcRn occurs in multiple places in humans: carrying IgG into the fetal bloodstream, in the adult liver, and from blood to milk in the lactating mammary glands and then by breast feeding into the gastrointestinal tract of the newborn. FcRn binds IgG at pH 6.0, which is the pH in the maternal bloodstream, and releases at pH 7.4, which is the pH in the fetal bloodstream. Trophoblast cells in the placenta uptake fluid-phase IgG by pinocytosis, maternal apical vesicles are released which are acidic and cause binding between FcRn and IgG. FcRn transports the antibodies across the cells, and upon reaching the fetal bloodstream where the IgG is released. FcRn then moves back across the trophoblast cells to the mother, to transcytose more IgG to the fetus (Simister, 1997).



**Fig.5.5c Perinatal maternal anti-allergic immunoprotection mechanism through IgG and TLRs (adaptive and innate maternal immunity).** The main function of Fc-receptors that signal through immunoreceptor tyrosine-based activation motifs (ITAMs) is to regulate signaling by Toll-like receptors (TLRs) under bacterial gears control (according to: Human Immune Protein FcRn: IgG Transcytosis and catabolism, 2003. Davidson College Biology – Immunology Course and completed with respect of the doctoral study concept)

The IgG-FcRn complex is sorted into vesicles from the apical endosome, and transported basolaterally to release IgG extracellularly. The FcRn controls the catabolism of IgG in maternal body, allowing IgG to be recycled to the cell surface and back into the bloodstream; therefore IgG has much longer half-life than other antibodies. The long intron of the human or mouse FcRn gene - FCGRT or Fcgrt - contains sequences that bind either p65/p50 heterodimers or p50/p50 homodimers of the NF- $\kappa$ B transcription factors. The presence of NF- $\kappa$ B binding sequences located in distant intronic regions involve the NF- $\kappa$ B complexes in the instruction of FcRn, possibly in cooperation with other transcriptional elements in the FcRn promoter.

Because the FcRn protein may exert both beneficial and detrimental effects in a variety of immune diseases, FcRn biosynthesis may be under the control of multiple complex regulatory mechanisms in response to an extracellular stimulus. This mechanism is common in cells which express FcR- and TLR receptors from innate and adaptive immunity. In general, the packaging of eukaryotic DNA into the nucleosome and higher order structures of chromatin allow for the possibility that the distant regulatory regions are physically close enough for direct interaction or communication through bridging molecules. It is tempting to speculate that the transcriptional factors binding to the proximal region of the FcRn promoter interact functionally with NF- $\kappa$ B dimer bound to the intronic sequences after induction by stimuli, such as TLR ligands. Thus, a protein complex is formed between an FcRn intron and an FcRn promoter region using a chromatin looping mechanism. Obviously, NF- $\kappa$ B is clearly involved in the transcriptional regulation of many genes. Its activity can be modulated significantly by factors that bind to motifs adjacent to, overlapping with or distant from that of NF- $\kappa$ B binding sequences. These protein-protein interactions may be involved in mediating the transcriptional regulation of the FcRn genes – FCGRT or Fcgrt - in response to stimuli and can functionally cooperate to elicit maximal activation of the promoter (Li, 2002; Hayden, 2004; Karin, 2000).



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**The role of Toll-like receptors in the development of immunological tolerance in neonates**

Epidemiological and clinical studies based on *Hygiene hypothesis* indicate that maternal exposure to bacterial environment may modulate allergic diseases later in life. Also, evidences suggest that priming of the immune responses against allergens happens already *in utero*. This is a *nature's lesson* taught by pregnancy immunity: maternal innate immunity via placental TLR signaling and adaptive immunity via maternal IgG and IgA transfer to fetus. The antibodies provide not only a passive protection, but are actively involved in the immunity of the child. Toll-like receptors (TLR) expressed on gut lymphoid tissue and epithelial cells of gastrointestinal-/respiratory tract, placenta etc. interact with environmental bacterial molecular patterns (e.g. endotoxin - LPS, DNA, LTA, dsRNA, ssRNA, flagellin etc.) and modulate maternal innate-/adaptive immune responses. In parallel, it also seems the T-cell system of the mother influences the development of children immune responses. Thus, in mother an appropriate colonization with probiotics helps to produce a balanced T helper cell response ( $T_H1/T_H2$ ). Nevertheless, the (maternal and infant) respiratory tract has an obvious relationship with the environment and continuous exposure to microbial environment. Regarding, an important aspect of the study was *to investigate the impact of non-pathogenic Gram<sup>+</sup> (LGG), Gram<sup>-</sup> bacteria (Ac.lwoffii) or bacterial gears, (LA/LPS/ E.coli) to allergic asthma*, and hypothesised that *the activation of Toll-like receptors in the placenta plays an important role in prenatal transmission of protective immunological effects from mother to fetus*. Consequently, the full development of innate host defense responses in the offspring lung requires expression of the maternal TLR in placenta. The receptors enhance expression of IL-1 $\beta$ , TNF $\alpha$ , IL-4, IL-10 and IFN $\gamma$ . A balance of  $T_H1/T_H2$  cytokine regulates the intensity and nature of the response. Maternal anti-allergic mechanism via TLR signaling is in general associated with the maturation and migration of the lung dendritic cells to the lymph nodes and production of  $T_H1$  cytokine (IL-12),

increasing the IFN $\gamma$  production either in the lung and in the early placenta. Thus, we associated suppression of allergen-mediated inflammatory responses in placenta with: a cross-talk between trophoblasts and activated lymphocytes, using TLR pathways by maintaining the T<sub>H</sub>1/T<sub>H</sub>2 balance pregnancy; secretion of immuno-suppressive molecules such as steroid hormones (progesterone, estradiol), prostaglandins, TGF $\beta$  by trophoblasts and decidual immune cells; a significant increase of placental mRNA TNF $\alpha$  and mRNA IL-1 $\beta$  cytokine, resulting in a down-regulation of mRNA IFN $\gamma$  expression under control of steroid hormones; passive blocking of lymphocyte activation by essential cytokine required for cell division, like IL-10; an immunoregulatory effect caused by the placental local production of specific sets of cytokine or chemokine, such as IL-4 and MCP-1; a specific cytostatic action of placenta CD8<sup>+</sup> Tc cells, where the trend appears dominant in supplemented group; decidual NK cells – high amount in the early pregnancy are reduced in the 3<sup>rd</sup> trimester being unable to produce IFN $\gamma$ , supporting the T<sub>H</sub>2 pregnancy phenotype and avoiding abortion; a strong induction of placental mRNA TLR4 and an oscillation in expression of mRNA TLR1, TLR2, TLR5, TLR7, TLR9 expression into Gram<sup>-</sup> bacteria/gears supplemented group; placental cytokine modulate the TLR gene expression, but the effect varied between different cell types; maternal antibodies transfer to the fetus (a mechanism via trophoblast's and gut's IgG-FcRn complex induced by TLR signaling cascade). Moreover, frequent perinatal bacterial exposure suppressed in susceptible offspring: the allergen-specific sensitization - IgE production; the numbers of inflammatory cells, such as eosinophils in BALF and T<sub>H</sub>2 cytokines (IL-4, IL-5, IL-13) expressed in BALF and serum; in vivo, airway reactivity in response to Methacholine; lung mucus production. There is no doubt that early programming contributes to a large extend to the development of a normal state of immunological responses in offspring. A diminished expression and function of maternal TLR may help explain the poor adaptive immune responses, increasing the susceptibility to infections and allergies in the offspring. However, once a pregnancy is well developed, the newborn has a

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weakly  $T_H2$ -biased immune response, and there is required a rapid postnatal down-regulated immunity. Therefore, the development of  $T_H1$  anti-allergic immunity in neonates, could be associated with the  $IFN\gamma$  compensation induced through a stable and specific perinatal non-pathogenic bacterial antigen exposure.

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### Die Rolle von Toll-like Rezeptoren bei der Entwicklung immunologischer Toleranz bei Neugeborenen

Epidemiologische und klinische Studien unterstützt durch die *Hygiene Hypothese* beweisen, dass die mütterliche Exposition gegenüber einer bakteriellen Umwelt allergische Erkrankungen im späteren Leben modulieren kann. Es ist nachgewiesen, dass die Ursachen dieser Immunantwort gegen Allergene bereits *in utero* zu finden sind. Das ist eine *Lektion der Natur*, die durch die Schwangerschaftsimmunität gelehrt wird: die angeborene (via Plazenta-TLR-Signalen) und die adaptive Immunität der Mutter. Die Antikörper bieten nicht nur einen passiven Schutz, sondern sind aktiv am Immunsystem des Kindes beteiligt. Toll-like Rezeptoren (TLR) wie z. B. am Darm-Lymphgewebe sowie den Epithelzellen des Magen-Darm-Trakts, der Atemwege und der Plazenta interagieren mit bakteriellen molekularen Mustern der Umwelt (z. B. Endotoxin-LPS, DNA, LTA, dsRNA, ssRNA, Flagellin etc.) und modulieren die angeborene und adaptive Immunantwort der Mutter. Parallel dazu scheint auch das T-Zell-System der Mutter die Entwicklung der kindlichen Immunantwort zu beeinflussen. So trägt eine geeignete Besiedelung der Mutter mit Probiotika dazu bei, eine ausgewogene T-Helferzellen-Antwort ( $T_H1/T_H2$ ) zu produzieren. Ein wichtiger Aspekt der Studie war es, *die Auswirkungen der nicht-pathogenen Gram<sup>+</sup> Bakterien (LGG), Gram<sup>-</sup> Bakterien (Ac.lwoffii) oder bakteriellen Komponenten (E.coli/LPS/LA) auf allergisches Asthma zu untersuchen*. Diesbezüglich wird die Hypothese aufgestellt, dass *die Aktivierung von Toll-like Rezeptoren in der Plazenta eine wichtige Rolle in der pränatalen Übertragung einer immunologischen Schutzwirkung von der Mutter zum Fötus spielt*. Folglich ist die Expression des mütterlichen TLR sowie die Fähigkeit, durch diese Rezeptoren Signale zu senden, für die volle Entfaltung der angeborenen Reaktionen des Abwehrsystems in der Lunge der Nachkommen erforderlich. Die Rezeptoren steigern die Expression von IL-1 $\beta$ , TNF $\alpha$ , IL-4, IL-10 und IFN $\gamma$ . Ein Gleichgewicht der  $T_H1$ - und  $T_H2$ -Zytokine reguliert die Intensität und Art der Immun-Antwort.

Dies bedeutet, dass – im Gegensatz zu den bekannten verschlimmernden Auswirkungen auf Asthma – die Exposition gegenüber Gram<sup>+</sup> Bakterien, Gram<sup>-</sup> Bakterien oder anderen TLR-Liganden in der frühen Kindheit Schutz übertragen oder zu einer Abnahme der Häufigkeit von Asthma im späteren Leben führen kann.

Der mütterliche Anti-Allergie-Mechanismus mittels TLR-Signalisierung wird allgemein mit der Reifung von dendritischen Zellen der Lunge und ihrer Migration zu den Lymphknoten sowie der Produktion von T<sub>H</sub>1-Zytokin (IL-12) verbunden, was zu einem Anstieg der IFN $\gamma$  Produktion in der Lunge und der frühen Plazenta führt. Auf diese Weise wurde der Zusammenhang zwischen der Unterdrückung der durch Allergene hervorgerufenen entzündlichen Reaktionen in der Plazenta und einer Reihe weiterer Phänomene festgestellt: *cross-talk*-Mechanismus zwischen Trophoblasten und aktivierten Lymphozyten, der die TLR-Signalwege unter Wahrung der Balance von T<sub>H</sub>1/T<sub>H</sub>2 in der Schwangerschaft verwendet; Sekretion von immunsuppressiven Molekülen, wie z. B. Steroidhormone (Progesteron, Estradiol), Prostaglandine, TGF $\beta$  durch Trophoblasten und Dezidua-Immunzellen; signifikante Zunahme der mRNA TNF und mRNA IL-1 $\beta$  Zytokinen in der Plazenta, was unter der Kontrolle von Steroidhormonen eine Unterdrückung der mRNA IFN $\gamma$  zur Folge hat; passives Blockieren der Lymphozyten-Aktivierung durch essentielle Zytokine, die für die Zellteilung erforderlich sind, wie z. B. IL-10; immunregulatorischer Effekt, ausgelöst durch die lokale Produktion von bestimmten Arten von Zytokinen oder Chemokinen, wie IL-4 und MCP-1, in der Plazenta; dominanter Trend in der behandelten Gruppe, vermutlich aufgrund einer spezifischen zytostatischen Wirkung der plazentaeigenen CD8<sup>+</sup> Tc-Zellen; deziduale NK-Zellen – sie sind in großer Menge in der frühen Schwangerschaft vorhanden und werden im 3. Trimester reduziert, da sie nicht in der Lage sind, IFN $\gamma$  zu produzieren – womit der T<sub>H</sub>2-Phänotyp der Schwangerschaft unterstützt und eine Fehlgeburt vermieden wird; starke Induktion der mRNA TLR4 Expression und variieren in der mRNA TLR1, TLR2, TLR5, TLR7, TLR9 Expression in der Plazenta, der mit Gram<sup>-</sup>

Bakterien/Komponenten behandelten Gruppe; Modulation der TLR-Genexpression erfolgt durch plazentaeigenes Zytokin, aber die Wirkung ist bei verschiedenen Zelltypen unterschiedlich; mütterliche Antikörper werden an den Fötus weitergegeben (ein Mechanismus, der über den FcRn IgG-Komplex der Trophoblasten und des Darms abläuft und durch eine TLR-Signalkaskade angestoßen wird).

Darüber hinaus unterdrückt häufige perinatale bakterielle Belastung der prädisponierten Nachkommen: die allergen-spezifische Sensibilisierung - IgE-Produktion; die Zahl der Entzündungszellen, wie Eosinophile in BALF und  $T_H2$ -Zytokinen (IL-4, IL-5, IL-13) exprimiert in BALF und Serum; in vivo Atemwegsreaktivität als Immunantwort auf Methacholine; die Produktion von Lungenschleim. Dennoch besteht kein Zweifel daran, dass eine frühe Programmierung in großem Maße zu der Entwicklung von einem normalen Zustand der Immunabwehr beiträgt.

Eine verminderte Expression und Funktion der mütterlichen TLR könnte zur Erklärung der geringen adaptiven Immunantworten beitragen, die die Anfälligkeit für Infektionen und Allergien der Nachkommen erhöhen. Sobald sich jedoch eine Schwangerschaft gut entwickelt hat, hat das Neugeborene eine schwach verzerrte  $T_H2$ -Immunantwort. Dann ist eine schnelle postnatale Unterdrückung der Immunität erforderlich. Daher könnte die Entwicklung von  $T_H1$ -Immunität bei Neugeborenen, einem Anti-Allergie-Phänotyp, mit dem IFN $\gamma$  Ausgleich zusammenhängen, der durch eine stabile und spezifische perinatale nicht-pathogene bakterielle Antigenbelastung angestoßen wird.

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**8.2 List of abbreviations**

$\alpha$ -GalCer	alfa-Galactosylceramide
$\alpha$ -FP	alfa-Fetoprotein
Ac. Iwoffii	Acinetobacter Iwoffii F78
AP	Alkaline Phosphatase
ALEX Study	Allergy and Endotoxin Study
AF	Amniotic Fluid
APC	Antigen Presenting Cell
B220	B cell isoform of 220 kDa expressed on B cells, activated T cells, a subset of dendritic cells and other APCs
BCA Kit	Bicinchoninic Acid assay (Kit)
BS	Blood Serum
BSA	Bovine Serum Albumin
BALF	Bronchoalveolar Lavage Fluid
CdC2	Cell division Cycle 2 protein
CSF-1	Colony Stimulating Factor-1
CSF-1	Colony Stimulating Factor-1
CFU	Colony Forming Unit
CRRY	Complement receptor 1-related gene/protein y
CD3	Cluster of Differentiation 3 for T-cell, co-receptor
CD4	Cluster of Differentiation 4 for T helper cells, regulatory T cells, monocytes, macrophages, and dendritic cells
CD8	Cluster of Differentition 8 for cytotoxic T cells and natural killer cells, co-receptor
CD11b	Cluster of Differentiation 11 for monocytes, granulocytes, macrophages and natural kiler cells, Integrin alpha M (ITGAM)

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CD11c	Cluster of Differentiation 11 for human dendritic cells, monocytes, macrophages, neutrophils and B cells, Integrin alpha X (complement component 3 receptor 4 subunit) (ITGAX)
CD25	Cluster of Differentiation 25 for T and B cells, co-receptor
CD45	Cluster of Differentiation 45 for Leukocyte Common Antigen (LCA)
CD69	Cluster of Differentiation 69, activates T lymphocytes
CD95	cluster of differentiation 95, Fas receptor (apoptosis)
CBA	Cytometric Bead Array
CpG	Cytosine – phosphate – Guanine
CTB	Cytotrophoblast
DAF	Decay Accelerating Factor
dDCs	decidual Dendritic Cells
DHEA-S	Dehydroepiandrosterone - sulphate
DC	Dendritic Cells
DNA	Deoxyribonucleic acid
DAB	3, 3'-Diaminobenzidine
dsRNA	double-stranded Ribonucleic Acid
EGF	Endothelial Growth Factor
ELISA	Enzyme-Linked ImmunoSorbent Assay
EtOH	Ethanol
EF <sub>50</sub>	(mid)-expiratory flow rate (50%)
EVT	Extravillous invasive Trophoblasts
FRT	Female Reproductive Tract
FCS	Fetal Calf Serum
FasL	Fas ligand
FasR	Fas receptor
FGF4	Fibroblast Growth Factor-4
Fgfr2	Fibroblast growth factor receptor 2
FITC	Fluorescein Isothiocyanate

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FACS	Fluorescent-Activated Cell Sorting (system)
FM	Fluorescent microscope
Foxp3	Forkhead-winged-helix protein 3
FcRn	Fragment cystallizable neonatal Receptor
Gcm1	Glial cells missing homolog 1 - transcription factor
gld mice	Glucose dehydrogenase gene deficient mice
GAPDH	Glycerin-Aldehyd-Phosphate Dehydrogenase
GPI	Glycosyl-Phosphatidyl-Inositol
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GALT	Gut-associated Lymphatic Tissue
H&E	Haematoxylin & Eosin (Staining)
HOBP	Head-out Body Plethysmograph
HRP	Horseradish peroxidase
HLA-G	Human Leukocyte Antigen-G
hCGH	human Chorionic Gonadotrophin Hormone
hPGH	human Placental Growth Hormone
hPLH	human Placental Lactogen Hormone
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IDO	Indoleamine 2,3-dioxygenase
iKIRs	inhibitory Killer Ig-like Receptors
ICM	Inner Cell Mass
IGF	Insuline like-Growth Factor
IGF-1	Insulin-like Growth Factor-1
IGF-BP-1	Insuline like-Growth Factor Binding Protein-1
IFN $\gamma$	Interferone gamma
IRF-3	Interferon Regulatory Factor 3
IRF-7	Interferon Regulatory Factor 7

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ISRE	Interferon Stimulated Response Element
IRAK-1	IL-1 Receptor Associated Kinase-1
IRAK-4	IL-1 Receptor Associated Kinase-4
IL-1	Interleukin-1
IL-1 $\beta$	Interleukin -1 beta
IL-1 RA	Interleukin -1 receptor antagonist
IL-11R	Interleukin –11 receptor
IL-12p70	Interleukin 12 heterodimer formed with IL-12p35
IUGR	Intrauterine Growth Restriction
J $\alpha$ 281	subtype of NK T cells (mouse)
LGG	Lactobacillus rhamnosus GG
LIF	Leukemia Inhibitory Factor
LIFR	Leukemia Inhibitory Factor Receptor
LM	Light microscope
LA	Lipid A
LTA	Lipoteichoic Acid
LBP	Lipopolysaccharide Binding Protein
LPS	Lipopolysaccharide
MD2	Lymphocyte antigen 96
mid-G1	Phase G1, protein synthesis in cell cycle
MACS	Magnetic-activated cell sorting
MHC	Major Histocompatibility Complex
MMPs	Matrix Metalloproteases
MMP-2	Matrix metalloproteinase-2
MCP-1	Membrane Cofactor Protein-1
MNCs	(splenic) mononuclear cells
MCh	$\beta$ -Methyl-Acetyl-Choline
MAL	MyD88-adaptor protein-like
MyD88	Myeloid Differentiation protein 88
NKT	Natural Killer T cells
NK1.1	Natural Killer cell surface marker 1.1 in mice

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NDV	Newcastle Disease Virus
NaCl	Sodium Chloride
OcT3	Organic cation Transporter 3
OVA	Ovalbumin
PAMPs	Pathogen Associated Molecular Patterns
PG	Peptidoglycan
Per CP	Peridinin Chlorophyll Protein
PAS	Periodic acid-Schiff
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PGH	Placental Growth Hormone
pDC	plasmacytoid Dendritic Cells
PAI-2	Plasminogen Activator Inhibitor-2
PAF	Platelet-Activating Factor
PCBs	Polychlorinated Biphenils
poly I:C	poly-Inosine:poly-Cytidilic acid
PVDF	Polyvinylidene difluoride
P	Progesterone
PGF $\alpha$ -2	Prostaglandin
PGE <sub>2</sub>	Prostaglandin
PIBF	Progesterone induced blocking factor
RIP1	Receptor Interacting Protein-1
RANTES	<i>Regulated upon Activation Normal T-cell Expressed and Secreted</i>
RNA	Ribonucleic acid
RPMI	“Roswell Park Memorial Institute” cell culture media
STAT4	Signal-Transducing Activator -4 of Transcription
STAT6	Signal-Transducing Activator -6 of Transcription
ssRNA	single-stranded Ribonucleic acid
sCD14	soluble CD14 (cluster of differentiation 14, cell surface marker protein, a pattern recognition receptor)

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sTLR2	soluble Toll-like receptor2
S-AP	Streptavidin-Alkaline Phosphatase
S-HRP	Streptavidin-Horseradish Peroxidase
TBK1	TANK Binding Kinase 1
TCR $\alpha$	T cell receptor alfa (complex)
TIRAP	TIR-Associated Protein
TICAM 1	TIR-domain Containing Adaptor Molecule 1
TRIF	TIR-domain containing adaptor protein inducing IFN- $\beta$
TIMPs	Tissue Inhibitor(s) of MMP(s)
TRAF-6	TNF Receptor Associated Factor-6
TLR	Toll-like receptors
TIR domain	Toll/IL1-Receptor like domain
TGF- $\alpha$ / TGF- $\beta$	Transforming Growth Factor -alfa and -beta
TRAM	TRIF-related Adaptor Molecule
TTBS	Tris-Buffered-Saline / Tween 20 mixtured buffer
TSCs	Trophoblast Stem Cells
TNF $\alpha$	Tumor Necrosis Factor alfa
uPA	urokinase Plasminogen Activator
uNKs	uterine Natural Killer cells
V $\alpha$ 14	subtype of Natural killer T cells (mouse)
VSV	Vesicular Stomatitis Virus
ZO-1	Zonula Occludens-1 protein
WB	Western Blot

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Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel *The role of Toll-like receptors in the development of immunological tolerance in neonates* in der Abteilung Klinische Chemie und Molekulare Diagnostik – BMFZ – Zentrallaboratorium der Phillips-Universität Marburg unter Leitung von Prof. Dr. med. H. Renz mit Unterstützung durch Prof. Dr. med. H. Renz ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

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(Cecilia-Carmen Patrascan)

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